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(54) *Viral vaccine for the prevention of porcine reproductive and respiratory syndrome*

(57) A virus has been isolated, capable of reproducing in sows the reproductive alterations associated with porcine reproductive and respiratory syndrome (PRRS) and in piglets, respiratory disorders, which may be used in the formulation of a vaccine capable of protecting sows against PRRS. A vaccine is described which contains a suitable quantity of viral antigen, inactivated, as well as a suitable adjuvant. The vaccine has proven to be efficacious in experiments of challenge with PRRS viruses different from the described Spanish strain. DNA sequences of Open Reading Frames 3 to 7 of the isolated virus, together with the corresponding amino acid sequences, are disclosed.

GB 2 282 811 A

ORF3

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ATG GCT CAT CAG TGT GCA CGC TTC CAT TTT TTC CTC TGT AGC TTC ATC     48
Met Ala His Gln Cys Ala Arg Phe His Phe Phe Leu Cys Ser Phe Ile
 1               5                  10                  15

TGT TAC CTT GTT CAT AGT GCT TTG GCT TCG AAT TCC AAT TCT ACG CTA     96
Cys Tyr Leu Val His Ser Ala Leu Ala Ser Asn Ser Asn Ser Thr Leu
            20                  25                  30

TGT TTT TGG TTT CCA TTG GCC CAC GGC AAC ACA TCA TTC GAG CTA ACC    144
Cys Phe Trp Phe Pro Leu Ala His Gly Asn Thr Ser Phe Glu Leu Thr
        35                  40                  45

ATC AAC TAC ACC ATA TGT ATG CCC TGC TCT ACC AGT CAA GCG GCT CAC    192
Ile Asn Tyr Thr Ile Cys Met Pro Cys Ser Thr Ser Gln Ala Ala His
    50                  55                  60

CAA AGA CTC GAG CCC GGT CGT AAC ATG TGG TGC AGA ATA GGG CAC GAC    240
Gln Arg Leu Glu Pro Gly Arg Asn Met Trp Cys Arg Ile Gly His Asp
65                  70                  75                  80

AGG TGT GAG GAA CGT GAC CAT GAT GAG TTG TCA ATG TCC ATT CCG TCT    288
Arg Cys Glu Glu Arg Asp His Asp Glu Leu Ser Met Ser Ile Pro Ser
                85                  90                  95

GGG TAC GAT AAC CTC AAA CTT GAG GGT TAT TAT GCT TGG CTG GCC TTT    336
Gly Tyr Asp Asn Leu Lys Leu Glu Gly Tyr Tyr Ala Trp Leu Ala Phe
            100                 105                 110

TTG TCC TTT TCC TAC GCG GCC CAA TTC CAT CCG GAG TTG TTC GGA ATA    384
Leu Ser Phe Ser Tyr Ala Ala Gln Phe His Phe Glu Leu Phe Gly Ile
        115                 120                 125

GGA AAC GTG TCG CGC GTC TTC GTG GAC AAG CAA CAC CAG TTC ATT TGC    432
Gly Asn Val Ser Arg Val Phe Val Asp Lys Gln His Gln Phe Ile Cys
    130                 135                 140

GCC GAG CAT GAT GGA CGA AAT TCA ACC ATA TCT ACC GAA TAT AAC ATC    480
Ala Glu His Asp Gly Arg Asn Ser Thr Ile Ser Thr Glu Tyr Asn Ile
145                 150                 155                 160

TCC GCA TTA TAT GCG TCG TAC TAC CAT CAC CAA ATA GAC GGG GGC AAC    528
Ser Ala Leu Tyr Ala Ser Tyr Tyr His His Gln Ile Asp Gly Gly Asn
                165                 170                 175

TGG TTC CAT TTG GTG CTC AAC ATT TCA GAA TGG CTG CGG CCA TTC TTT    576
Trp Phe His Leu Glu Trp Leu Arg Pro Phe Phe Ser Ser Trp Leu Val
            180                 185                 190

TCC TCC TGG CTG TGG TTT CTG AGG CGT TCG CCT GTA AGC CCT GTT TCT    624
Leu Asn Ile Ser Trp Phe Leu Arg Arg Ser Pro Val Ser Pro Val Ser
        195                 200                 205
```

FIGURE 1

```
CGA CGC ATC TAT CAG ATA TTA AGA CCA ACA CGA CCG CGG CTG CCG GTT   672
Arg Arg Ile Tyr Gln Ile Leu Arg Pro Thr Arg Pro Arg Leu Pro Val
    210                 215                 220

TCA TGG TCC TTC AGA ACA TCA ATT GTC TCC GAC CTC ACG GGG TCT CAA   720
Ser Trp Ser Phe Arg Thr Ser Ile Val Ser Asp Leu Thr Gly Ser Gln
225                 230                 235                 240

CAG CGC AAG AGA ACA TTT CCT TCG GGA AGC CGT CTC AAT GTC GTG AAG   768
Gln Arg Lys Arg Thr Phe Pro Ser Gly Ser Arg Leu Asn Val Val Lys
                245                 250                 225

CCG TCG GTA TTC CCC AGT ACA TTA CGA TAA                         798
Pro Ser Val Phe Pro Ser Thr Leu Arg End
            260                 265
```

FIGURE 1 (Cont.)

ORF 4

```
ATG GCT GCG GCC ATT CTT TTC CTC CTG GCT GGT GCT CAA CAT TTC ATG   48
Met Ala Ala Ala Ile Leu Phe Leu Leu Ala Gly Ala Gln His Phe Met
 1               5                  10                  15

GTT TCT GAG GCG TTC GCC TGT AAG CCC TGT TTC TCG ACG CAT CTA TCA   96
Val Ser Glu Ala Phe Ala Cys Lys Pro Cys Phe Ser Thr His Leu Ser
            20                  25                  30

GAT ATT AAG ACC AAC ACG ACC GCG GCT GCC GGT TTC ATG GTC CTT CAG  144
Asp Ile Lys Thr Asn Thr Thr Ala Ala Ala Gly Phe Met Val Leu Gln
        35                  40                  45

AAC ATC AAT TGT CTC CGA CCT CAC GGG GTC TCA ACA GCG CAA GAG AAC  192
Asn Ile Asn Cys Leu Arg Pro His Gly Val Ser Thr Ala Gln Glu Asn
    50                  55                  60

ATT TCC TTC GGG AAG CCG TCT CAA TGT CGT GAA GCC GTC GGT ATT CCC  240
Ile Ser Phe Gly Lys Pro Ser Gln Cys Arg Glu Ala Val Gly Ile Pro
65                  70                  75                  80

CAG TAC ATT ACG ATA ACG GCT AAT GTG ACC GAT GAA TCG TAT TTG TAC  288
Gln Tyr Ile Thr Ile Thr Ala Asn Val Thr Asp Glu Ser Tyr Leu Tyr
                85                  90                  95

AAC GCG GAC TTG CTG ATG CTT TCT GCG TGC CTT TTC TAC GCT TCA GAA  336
Asn Ala Asp Leu Leu Met Leu Ser Ala Cys Leu Phe Tyr Ala Ser Glu
            100                 105                 110

ATG AGC GAA AAA GGC TTC AAA GTT ATC TTT GGG AAC GTC TCT GGC GTT  384
Met Ser Glu Lys Gly Phe Lys Val Ile Phe Gly Asn Val Ser Gly Val
        115                 120                 125

GTT TCT GCT TGT GTC AAT TTT ACA GAT TAT GTG GCC CAT GTG ACC CAA  432
Val Ser Ala Cys Val Asn Phe Thr Asp Tyr Val Ala His Val Thr Gln
    130                 135                 140

CAT ACC CAG CAG CAT CAT CTG GTA ATT GAT CAC ATT CGG TTG CTG CAT  480
His Thr Gln Gln His His Leu Val Ile Asp His Ile Arg Leu Leu His
145                 150                 155                 160

TTC TTG ACA CCA TCT ACA ATG AGG TGG GCT ACA ACC ATT GCT TGT TTG  528
Phe Leu Thr Pro Ser Thr Met Arg Trp Ala Thr Thr Ile Ala Cys Leu
                165                 170                 175

TTC GCC ATT CTC TTG GCG ATA TGA                                 552
Phe Ala Ile Leu Leu Ala Ile End
            180
```

FIGURE 2

ORF 5

```
ATG AGA TGT TCT CAC AAA TTG GGG CGT TTC TTG ACT CCT CAC TCT TGC   48
Met Arg Cys Ser His Lys Leu Gly Arg Phe Leu Thr Pro His Ser Cys
  1               5                  10                  15

TTC TGG TGG CTT TTT TTG CTG TGT ACC GGC TTG TCC TGG TCC TTT GTC   96
Phe Trp Trp Leu Phe Leu Leu Cys Thr Gly Leu Ser Trp Ser Phe Val
            20                  25                  30

GCT GGC GGC AGC AGC TCG ACA TAC CAA TAC ATA TAT AAC TTA ACG ATA  144
Ala Gly Gly Ser Ser Ser Thr Tyr Gln Tyr Ile Tyr Asn Leu Thr Ile
        35                  40                  45

TGC GAG CTG AAT GGG ACC GAC TGG TTG TCC AAC CAT TTT GAT TGG GCA  192
Cys Glu Leu Asn Gly Thr Asp Trp Leu Ser Asn His Phe Asp Trp Ala
    50                  55                  60

GTC GAG ACC TTT GTG CTT TAC CCG GTT GCC ACT CAT ATC CTC TCA CTG  240
Val Glu Thr Phe Val Leu Tyr Pro Val Ala Thr His Ile Leu Ser Leu
65                  70                  75                  80

GGT TTT CTC ACA ACA AGC CAT TTT TTT GAC GCG CTC GGT CTC GGC GCT  288
Gly Phe Leu Thr Thr Ser His Phe Phe Asp Ala Leu Gly Leu Gly Ala
                85                  90                  95

GTG TCC ACT ATA GGA TTT GTT GGC GGG CGG TAT GTA CTC AGC AGC GTG  336
Val Ser Thr Ile Gly Phe Val Gly Gly Arg Tyr Val Leu Ser Ser Val
            100                 105                 110

TAC GGC GCT TGT GCT TTC GCA GCG TTC GTA TGT TTT GTC ATC CGT GCT  384
Tyr Gly Ala Cys Ala Phe Ala Ala Phe Val Cys Phe Val Ile Arg Ala
        115                 120                 125

GTT AAA AAT TGC ATG GCT TGC CGC TAT GCC CAC ACC CGG TTT ACC AAC  432
Val Lys Asn Cys Met Ala Cys Arg Tyr Ala His Thr Arg Phe Thr Asn
    130                 135                 140

TTC ATT GTG GAC GAC CGG GGG AGA ATC CAT CGG TGG AAG TCT CCA ATA  480
Phe Ile Val Asp Asp Arg Gly Arg Ile His Arg Trp Lys Ser Pro Ile
145                 150                 155                 160

GTG GTA GAG AAA TTG GGC AAA GCT GAA GTC GGT GGC GAC CTT GTC ACC  528
Val Val Glu Lys Leu Gly Lys Ala Glu Val Gly Gly Asp Leu Val Thr
                165                 170                 175

ATC AAA CAT GTC GTC CTC GAA GGG GTT AAA GCT CAA CCC TTG ACG AGG  576
Ile Lys His Val Val Leu Glu Gly Val Lys Ala Gln Pro Leu Thr Arg
            180                 185                 190

ACT TCG GCT GAG CAA TGG GAA GCC TAG                              603
Thr Ser Ala Glu Gln Trp Glu Ala End
        195                 200
```

FIGURE 3

ORF 6

```
ATG GGA AGC CTA GAC GAT TTT TGC AAT GAT TCT ACC GCC GCA CAA AAG   48
Met Gly Ser Leu Asp Asp Phe Cys Asn Asp Ser Thr Ala Ala Gln Lys
  1               5                  10                  15

CTT GTG CTA GCC TTT AGC ATT ACA TAT ACA CCT ATA ATG ATA TAC GCC   96
Leu Val Leu Ala Phe Ser Ile Thr Tyr Thr Pro Ile Met Ile Tyr Ala
            20                  25                  30

CTT AAG GTG TCA CGC GGC CGA CTC CTG GGG CTG TTG CAC ATC CTA ATA  144
Leu Lys Val Ser Arg Gly Arg Leu Leu Gly Leu Leu His Ile Leu Ile
        35                  40                  45

TTC CTG AAT TGT TCT TTC ACA TTC GGA TAC ATG ACA TAT GTG CGT TTT  192
Phe Leu Asn Cys Ser Phe Thr Phe Gly Tyr Met Thr Tyr Val Arg Phe
    50                  55                  60

CAA TCC ACC AAC CGT GTC GCA CTT ACT CTG GGG GCT GTT GTC GCC CTT  240
Gln Ser Thr Asn Arg Val Ala Leu Thr Leu Gly Ala Val Val Ala Leu
65                  70                  75                  80

CTG TGG GGT GTT TAC AGC TTC ACA GAG TCA TGG AAG TTT GTT ACT TCC  288
Leu Trp Gly Val Tyr Ser Phe Thr Glu Ser Trp Lys Phe Val Thr Ser
                85                  90                  95

AGA TGC AGA TTG TGT TGC CTA GGC CGG CGA TAC ATT CTG GCC CCT GCC  336
Arg Cys Arg Leu Cys Cys Leu Gly Arg Arg Tyr Ile Leu Ala Pro Ala
            100                 105                 110

CAT CAC GTA GAA AGT GCT GCA GGT CTC CAT TCA ATC CCA GCG TCT GGT  384
His His Val Glu Ser Ala Ala Gly Leu His Ser Ile Pro Ala Ser Gly
        115                 120                 125

AAC CGA GCA TAC GCT GTG AGA AAG CCC GGA CTA ACA TCA GTG AAC GGC  432
Asn Arg Ala Tyr Ala Val Arg Lys Pro Gly Leu Thr Ser Val Asn Gly
    130                 135                 140

ACT CTA GTT CCA GGA CTT CGG AGC CTC GTG CTG GGC GGC AAA CGA GCT  480
Thr Leu Val Pro Gly Leu Arg Ser Leu Val Leu Gly Gly Lys Arg Ala
145                 150                 155                 160

GTT AAA CGA GGA GTG GTT AAC CTC GTC AAG TAT GGC CGG TAA          522
Val Lys Arg Gly Val Val Asn Leu Val Lys Tyr Gly Arg End

                165                 170
```

FIGURE 4

ORF 7

```
ATG GCC GGT AAA AAC CAG AGC CAG AAG AAA AAG AAA AGT GCA GCT CCG     48
Met Ala Gly Lys Asn Gln Ser Gln Lys Lys Lys Lys Ser Ala Ala Pro
  1               5                  10                  15

ATG GGG AAT GGC CAG CCA GTC AAT CAA CTG TGC CAG TTG CTG GGT GCA     96
Met Gly Asn Gly Gln Pro Val Asn Gln Leu Cys Gln Leu Leu Gly Ala
            20                  25                  30

ATG ATA AAG TCC CAG CGC CAG CAA CCT AGG GGA GGA CAG GCC AAA AAG    144
Met Ile Lys Ser Gln Arg Gln Gln Pro Arg Gly Gly Gln Ala Lys Lys
        35                  40                  45

AAA AAG CCT GAG AAG CCA CAT TTT CCC TTA GCT GCT GAA GAT GAC ATC    192
Lys Lys Pro Glu Lys Pro His Phe Pro Leu Ala Ala Glu Asp Asp Ile
    50                  55                  60

CGG CAC CAC CTC ACC CAG ACC GAA CGT TCC CTC TGC TTG CAA TCG ATC    240
Arg His His Leu Thr Gln Thr Glu Arg Ser Leu Cys Leu Gln Ser Ile
65                  70                  75                  80

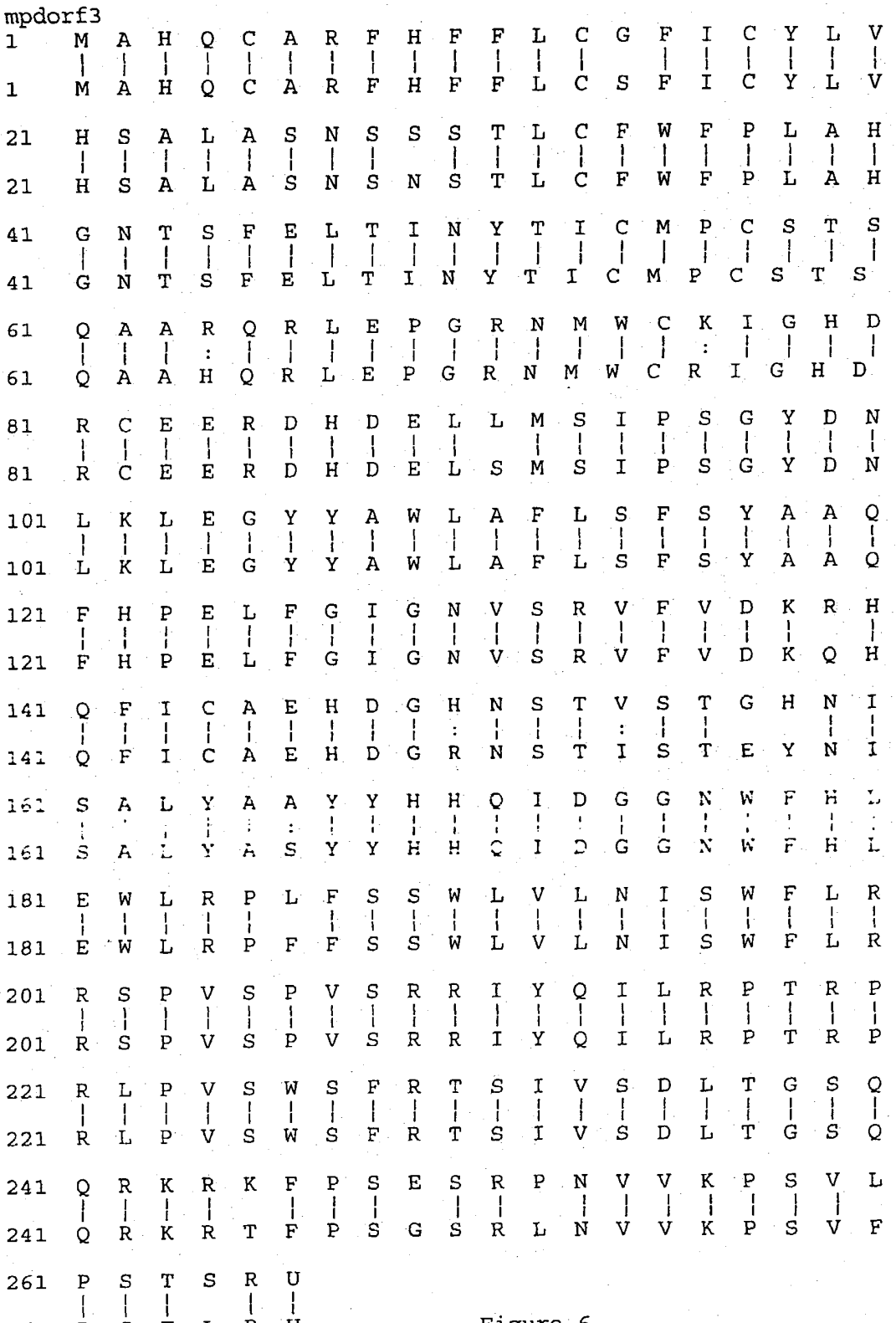
CAG ACG GCT TTT AAT CAA GGC GCA GGA ACT GCG TCG CTT TCA TCC AGC    288
Gln Thr Ala Phe Asn Gln Gly Ala Gly Thr Ala Ser Leu Ser Ser Ser
                85                  90                  95

GGG AAG GTC AGT TTT CAG GTT GAG TTC ATG CTG CCG GTT GCT CAT ACG    336
Gly Lys Val Ser Phe Gln Val Glu Phe Met Leu Pro Val Ala His Thr
            100                 105                 110

GTG CGC CTG ATT CGC GTG ACT TCT ACA TCC GCC AGT CAG GGT GCA AGC    384
Val Arg Leu Ile Arg Val Thr Ser Thr Ser Ala Ser Gln Gly Ala Ser
        115                 120                 125

TAA                                                                387
End
```

FIGURE 5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mpdorf3
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21 H S A L A S N S N S T L C F W F P L A H
41 G N T S F E L T I N Y T I C M P C S T S
41 G N T S F E L T I N Y T I C M P C S T S
61 Q A A R Q R L E P G R N M W C K I G H D
61 Q A A H Q R L E P G R N M W C R I G H D
81 R C E E R D H D E L L M S I P S G Y D N
81 R C E E R D H D E L S M S I P S G Y D N
101 L K L E G Y Y A W L A F L S F S Y A A Q
101 L K L E G Y Y A W L A F L S F S Y A A Q
121 F H P E L F G I G N V S R V F V D K R H
121 F H P E L F G I G N V S R V F V D K Q H
141 Q F I C A E H D G H N S T V S T G H N I
141 Q F I C A E H D G R N S T I S T E Y N I
161 S A L Y A A Y Y H H Q I D G G N W F H L
161 S A L Y A S Y Y H H Q I D G G N W F H L
181 E W L R P L F S S W L V L N I S W F L R
181 E W L R P F F S S W L V L N I S W F L R
201 R S P V S P V S R R I Y Q I L R P T R P
201 R S P V S P V S R R I Y Q I L R P T R P
221 R L P V S W S F R T S I V S D L T G S Q
221 R L P V S W S F R T S I V S D L T G S Q
241 Q R K R K F P S E S R P N V V K P S V L
241 Q R K R T F P S G S R L N V V K P S V F
261 P S T S R U
261 P S T L R U

Figure 6

lelorf4
mpdorf4

```
1    M A A A T L F F L A G A Q H I M V S E A
     | | | |   | |   | | | | | |   | | | | |
1    M A A A I L F L L A G A Q H F M V S E A

21   F A C K P C F S T H L S D I E T N T T A
     | | | | | | | | | | | | |   | | | | | |
21   F A C K P C F S T H L S D I K T N T T A

41   A A G F M V L Q D I N C F R P H G V S A
     | | | | | | | |   | | |   | | | | | | :
41   A A G F M V L Q N I N C L R P H G V S T

61   A Q E K I S F G K S S Q C R E A V G T P
     | | |   | | | | | |   | | | | | | |   |
61   A Q E N I S F G K P S Q C R E A V G I P

81   Q Y I T I T A N V T D E S Y L Y N A D L
     | | | | | | | | | | | | | | | | | | |
81   Q Y I T I T A N V T D E S Y L Y N A D L

101  L M L S A C L F Y A S E M S E K G F K V
     | | | | | | | | | | | | | | | | | | |
101  L M L S A C L F Y A S E M S E K G F K V

121  I F G N V S G V V S A C V N F T D Y V A
     | | | | | | | | | | | | | | | | | | |
121  I F G N V S G V V S A C V N F T D Y V A

141  H V T Q H T Q Q H H L V I D H I R L L H
     | | | | | | | | | | | | | | | | | | |
141  H V T Q H T Q Q H H L V I D H I R L L H

161  F L T P S A M R W A T T I A C L F A I L
     | | | | | : | | | | | | | | | | | | |
161  F L T P S T M R W A T T I A C L F A I L

181  L A I U
     | | | |
181  L A I U
```

Figure 7

lelorf5
mpdorf5

```
1    M R C S H K L G R F L T P H S C F W W L
     | | | | | | | | | | | | | | | | | | |
1    M R C S H K L G R F L T P H S C F W W L

21   F L L C T G L S W S F A D G N G D S S T
     | | | | | | | | | | |     |   | : | | |
21   F L L C T G L S W S F V A G   G S S S T

41   Y Q Y I Y N L T I C E L N G T D W L S S
     | | | | | | | | | | | | | | | | | |
40   Y Q Y I Y N L T I C E L N G T D W L S N

61   H F G W A V E T F V L Y P V A T H I L S
     | |   | | | | | | | | | | | | | | | |
60   H F D W A V E T F V L Y P V A T H I L S

81   L G F L T T S H F F D A L G L G A V S T
     | | | | | | | | | | | | | | | | | |
80   L G F L T T S H F F D A L G L G A V S T

101  A G F V G G R Y V L C S V Y G A C A F A
       | | | | | | | | |   | | | | | | | |
100  I G F V G G R Y V L S S V Y G A C A F A

121  A F V C F V I R A A K N C M A C R Y A R
     | | | | | | | | |   | | | | | | | | :
120  A F V C F V I R A V K N C M A C R Y A H

141  T R F T N F I V D D R G R V H R W K S P
     : | | | | | | | | | | : | | : . .
140  T K F T N F I V D D R G R I H R W K S P

161  I V V E K L G K A E V D G N L V T I K H
     : | | | | | | | | | | | | | | | | |
160  I V V E K L G K A E V G G D L V T I K H

181  V V L E G V K A Q P L T R T S A E Q W E
     | | | | | | | | | | | | | | | | | |
180  V V L E G V K A Q P L T R T S A E Q W E

201  A U
     | |
200  A U
```

Figure 8

lelorf6
mpdorf6

```
1    M G G L D D F C N D P I A A Q K L V L A
     | | | | | | | | | | | | | | | | | | |
1    M G S L D D F C N D S T A A Q K L V L A

21   F S I T Y P T I M I Y A L K V S R G R L
     | | | | | | | | | | | | | | | | | | |
21   F S I T Y T P I M I Y A L K V S R G R L

41   L G L L H I L I F L N C S F T F G Y M T
     | | | | | | | | | | | | | | | | | | |
41   L G L L H I L I F L N C S F T F G Y M T

61   Y V H F Q S T N R V A L T L G A V V A L
     | | : | | | | | | | | | | | | | | | |
61   Y V R F Q S T N R V A L T L G A V V A L

81   L W G V Y S F T E S W K F I T S R C R L
     | | | | | | | | | | | | : | | | | | |
81   L W G V Y S F T E S W K F V T S R C R L

101  C C L G R R Y I L A P A H H V E S A A G
     | | | | | | | | | | | | | | | | | | |
101  C C L G R R Y I L A P A H H V E S A A G

121  L H S I S A S G N R A Y A V R K P G L T
     | | | | | | | | | | | | | | | | | | |
121  L H S I P A S G N R A Y A V R K P G L T

141  S V N G T L V P G L R S L V L G G K R A
     | | | | | : | | | | | | | | | | | | |
141  S V N G T L V P G L R S L V L G G K R A

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     | | | | | | | | | | | | |
161  V K R G V V N L V K Y G R U
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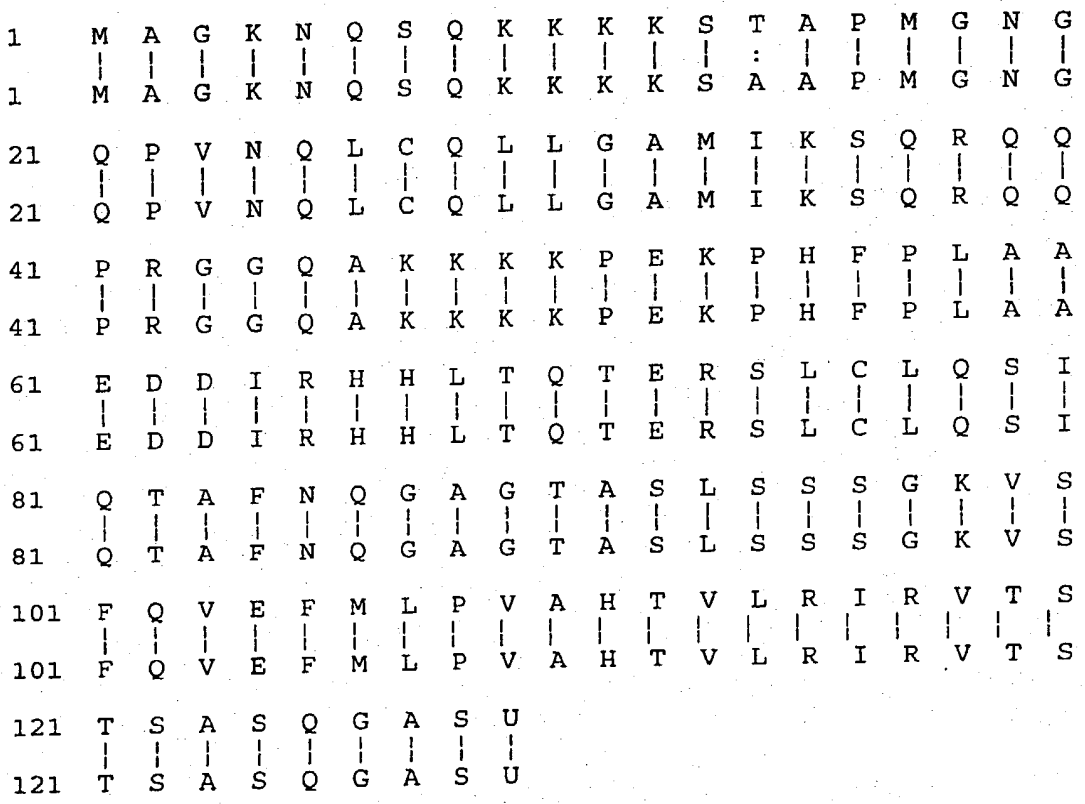
Figure 9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61 E D D I R H H L T Q T E R S L C L Q S I
61 E D D I R H H L T Q T E R S L C L Q S I
81 Q T A F N Q G A G T A S L S S S G K V S
81 Q T A F N Q G A G T A S L S S S G K V S
101 F Q V E F M L P V A H T V L R I R V T S
101 F Q V E F M L P V A H T V L R I R V T S
121 T S A S Q G A S U
121 T S A S Q G A S U

Figure 10

VACCINE FOR THE PREVENTION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME

SCOPE OF THE INVENTION

This invention relates to a vaccine capable of preventing porcine reproductive and respiratory syndrome, in particular, to an inactivated vaccine containing the causative virus of the said disease in inactivated form.

BACKGROUND OF THE INVENTION

The disease known as porcine reproductive and respiratory syndrome (PRRS) affects pregnant sows in which it can provoke anorexia, abortions, stillbirths, mummified fetuses, weak piglets that die in a few hours or days of life, respiratory post farrowing problems and breeding problems (Loula, T., "Clinical Presentation of Mystery Pig Disease in the breeding herd and suckling piglets", Proceedings of the Mystery Swine Disease Committee Meeting, October 6, 1990, Denver, Colorado, Livestock Conservation Institute, Madison, WI, (USA). Some cases have been described in which infected sows present blue spots on the ears, for which reason the disease has also been known as "Blue abortion", or "Blue-eared pig disease" (Veterinary Record, Vol. 130, no. 3, January 18, 1992). Other names given to the disease are "Mystery Pig Disease" (MPD), "Mystery Swine Disease" (MSD), "Mysterious Reproductive Syndrome" (MRS), "Swine Infertility and Respiratory Syndrome (SIARS) or "Porcine Epidemic Abortion and Respiratory Syndrome" (PEARS).

The first epizootic outbreaks of this disease appeared in the United States and Canada in 1987. In Europe, the first outbreak was detected in Germany in 1990, from where it spread to the Netherlands and Belgium late in 1990 and early in 1991. In Spain, the first cases of the disease were detected in mid-

January 1991, when important respiratory alterations were observed in a 300-piglet batch imported from Germany (Plana et al., Med. Vet., Vol. 8, no. 11, 1991). Shortly afterwards, in two breeding herds that were situated 500 meters from the herd where the initial problem had appeared, a disease was detected characterized by an abnormally high number of abortions during the last phase of gestation, as well as 70% mortality in piglets. Analysing the observed clinical signs, and bearing in mind that (i) these herds were subjected to an intensive vaccination program against porcine parvovirosis, Aujeszky's disease and swine influenza, and that (ii) laboratory trials had discarded the presence of other abortive diseases, the clinical presence of PRRS was suspected. In samples from these farms the causative agent of the disease has been isolated, as described in further detail below.

A number of agents have been correlated with this infectious process, among which are: encephalomyocarditis virus, swine influenza, classic swine fever, African swine fever, mucosal disease, Aujeszky's disease, brucellosis, leptospirosis, Q fever, parvovirosis and chlamydia disease, although some authors have also related it to mycotoxins (Loula, T., "Clinical Presentation of Mystery Pig Disease in the breeding herd and suckling piglets", Proceedings of the Mystery Swine Disease Committee Meeting, supra, Mengeling, W.L. and Lager, K.M., "Mystery Pig Disease: Evidence and considerations for its etiology", in Proceedings of the Mystery Swine Disease Committee Meeting, supra; Dea et al., "Virus isolation from farms in Quebec experiencing severe outbreaks of respiratory and reproductive problems", Proceedings of the Mystery Swine Disease Committee Meeting, supra; Van Alstine, W., "Past diagnostic approaches and findings and potential useful diagnostic strategies", in Proceedings of the Mystery Swine Disease Committee Meeting, supra; Loula, T. Agri-Practice, 12(1): 23-33, 1991; Woolen, N. et al., J. Am. Vet. Assoc. 197:600-601, 1990).

The PCT patent request published under publication number

WO 92/21375, in the name of Stitching Centraal Diergeneeskundig Instituut (CDI), describes the isolation of a virus denominated "Lelystad Agent" (LA) or "Lelystad Virus (LV) which is identified by them as the causative agent of the disease denominated at that time as MSD. The LV isolated when inoculated intranasally in pregnant sows produces loss of appetite and even refusal to ingest during days 4 to 10-12 post inoculation, reproductive disorders, and a blue colouring on the ears of some of the infected sows of days 9-10 post inoculation. However, it is also observed that in 2 of the 8 experimentally infected sows the disease does not reproduce (see Table 6 of the said PCT request), since in one of the sows the number of piglets born alive and surviving the first week is large (6 out 9, sow 1305) and another sow had two stillborn piglets whereas the other 9 survived the first week (sow 1065). The LV virus isolated by CDI belongs to the Arteriviridae genus, has a genome made up of one polyadenylated RNA molecule 14.5 to 15.5 kb in length (determined in neutral agarose gel), which replicates by means of a set of subgenomic RNAs at the 3' end. The above-indicated PCT request shows the LV genome nucleotide sequence, the 8 possible open reading frames (ORF), the amino acid sequence deduced from the identified ORFs and the putative sites of N-glycosilation. Posteriorly, other viruses causative of PRRS have been isolated in German, French and Spanish farms. The virus isolated in Tubingen, Germany (TV) [(Virology, 193, 329-339 (1993)] presents 99.3% homology to LV at the nucleotide level contained in ORFs 2 to 7 (there are 24 different base pairs (bp) out of the total of 3316 bp included in that region). The deduced TV amino acid sequence presents 99.2% homology to LV (there are only 10 different amino acids in the deduced amino acid sequences coded by ORFs 2 to 5, since there are no differences in the sequences deduced from ORFs 6 and 7).

On the other hand, the homology found between LV and TV against the virus isolated at our laboratory (Spanish strain or SV) is smaller. Up till now, only the nucleotide and amino acid sequences corresponding to ORFs 3 to 7 have been compared, with

the following results:

1) There is 95.5% homology at nucleotide level of SV in front of LV or TV (out of a total of 2599bp there are 144 that are different); and

2) There is 94.9% homology at amino acid level of SV in front of LV or TV (out of a total of 955 amino acids, a total of 47 amino acids are observed to be different).

These amino acid variations may be related to the higher pathogenicity of a strain in comparison with another, for reason that the virus isolated at our laboratories (Spanish strain) is more pathogenic than other known PRRS viruses. For example, the virus isolated in France (French strain), as shown in Example 8 of this description, where it can be seen that the percentage of piglets born alive to a sow infected with the French strain is 75% whereas the percentage is 9.5% when sows are infected with the Spanish strain (Tables 12 and 14). The percentage is 61% when sows are infected with LV (Table 6 of the PCT request no. WO 92/21375) since 58 piglets were born alive out of a total of 95 piglets.

The disease causes severe losses to the porcine industry as it can provoke, in acute outbreaks, mortality of 70% of the piglets in a litter. A means to solve the problem created would be to conduct a suitable vaccination program that would allow the prevention of the appearance of the disease. To that end, vaccines, capable of bringing about effective prevention of PRRS would be required.

The PCT patent request published under no. WO 93/06211 in the name of Collins, J.E., and Benfield, D.A., refers to a vaccine against MSD containing an infectious agent isolated from lungs of pigs infected with MSD. However, the said PCT request does not describe the characterization or identification of the infectious agent, nor has it been deposited to any Authorized

Deposit Institution. For all this, the realization of the knowledge derived from that PCT request presents serious reproducibility problems because the product described in the PCT request can not be verified nor the obtained results compared with those obtained by the petitioners of the PCT. Likewise, in this PCT request are not clearly expressed the antigen nor the adjuvant used in the formulation of the vaccine which, as will be verified posteriorly, play a very important part, nor are examples described that demonstrate the potency and efficacy of the said vaccines.

The PCT request published under no. WO 93/07898 refers to, among other things, the identification of the agent causative of PRRS and to vaccines derived from it. The isolated virus has characteristics similar to LV but, differently from the virus isolated at our laboratory, it is not capable of growing on ST cells (swine testis cells) at detectable levels. Apparently, the vaccine has been efficacious, but the level of protection has only been effective in approximately 52% of vaccinated animals only, which can be considered as a relatively low level of protection, especially if we bear in mind the high viral titre employed in the vaccinal dose. Additionally, this PCT request does not afford any information on the organization of the virus genome nor on the proteins coded for which reason the comparison between this virus and the virus obtained at our laboratories cannot be carried out or may be carried out but by exerting an undue research effort. Therefore, the need for vaccines capable of preventing PRRS continues to exist. In order to solve this problem, this invention provides a vaccine capable of efficaciously protecting sows against the infectious disease. The antigenic phase of this vaccine is composed of an inactivated Spanish isolate. The invention also provides combinations of the isolated PRRS viral antigen (Spanish strain) together with different porcine pathogens with the purpose of providing bi- or multivalent vaccines.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1 to 5 show the cDNA sequences corresponding to ORFs 3 to 7, respectively, of the PRRS virus (Spanish strain) as well as the sequence deduced from amino acids coded by each ORF.

Figures 6 to 10 show the homology and existing differences between LV and the virus isolated at our laboratories, at the level of amino acid sequences deduced from ORFs 3 to 7. The amino acids are expressed in accordance with a one letter code. The upper line of each two lines corresponds to the LV amino acid sequence, whereas the lower line corresponds to the virus isolated at our laboratories (Spanish strain). The homologous amino acids are represented by two vertical lines, while when there are substitutions of some amino acids by others they are represented by two vertical dots in cases of conservative substitutions, that is the substitution of an amino acid by another one functionally equivalent. The absence of vertical lines and dots between two amino acids represents the existence of non-conservative substitutions.

DETAILED DESCRIPTION OF THE INVENTION

1. Samples

1.1 Animals chosen for the isolation of the virus

Mummified fetuses, stillborn piglets and living but weak piglets of 1 to 10 days of age, progeny of field sows with clinical problems due to PRRS, were chosen.

1.2 Preparation of the samples

Lung, spleen, liver, kidney, brain and heart samples from the piglets were obtained by means of necropsy. In one

particular case, samples were prepared from the lung of a stillborn piglet born to a sow suspected of being affected with PRRS. With that lung, a homogenate was prepared with DMEM (GIBCO), (10 g of lung in 90 ml DMEM) supplemented with a solution of antibiotics (PEG) composed of 1000 IU/ml of penicillin, 1 mg/ml of streptomycin and 0.5 mg/ml of gentamicin. The resulting suspension was allowed to stand for 1 hour at 4°C, was frozen and thawed twice, centrifuged and the supernatant obtained stored at -70°C to be used in the infection of pig's lung alveolar macrophages. In another case lung samples were prepared from a piglet born alive but which died within a few hours after birth, by means of a similar process.

Additionally, blood was extracted from the animals by puncturing the vena cava to obtain (i) blood plasma, which was stored at -70°C and used for virus isolation, and (ii) serum, which was used to carry out antibody titration.

2. Pig's lung alveolar macrophages

2.1 Obtainment

Pigs seronegative to Aujeszky's disease, porcine parvovirosis, foot-and-mouth disease, classic swine fever, swine influenza (types H1N1 and H3N2) and transmissible gastroenteritis were used. The age of the pigs used ranged between 7 and 8 weeks. The animals were anaesthetized with phenobarbital sodium before the extraction of the lungs, which was done by first ligating the trachea below the epiglottis and by sectioning above the ligature. Once the lung had been extracted, it was washed externally with physiological saline solution, and then PBS and PEG solution of antibiotics were introduced by means of successive washings. The cells obtained from these washings were subjected to centrifugation for 10 minutes at 300g and resuspended in DMEMs medium [(DMEM medium supplemented with unessential amino acids (GIBCO), 1% of sodium pyruvate 1 mM and 1% of glutamine 2mM)], 10% fetal calf serum (FCS) and PEG

solution of antibiotics at 1%. The cell count was done in Newbauer chambers.

2.2 Sterility Control

It is verified that the pig's lung alveolar macrophages are free of contamination by bacteria, fungi and other viruses, by means of the suitable tests. Likewise the good general state of the cells is verified by optical microscopy.

The absence of contamination by mycoplasmas is also verified by cytochemical detection with DAPI (4.6-diamidine-2-phenyl-indole) which binds selectively to DNA and forms DNA-DAPI complexes of high fluorescence and high specificity.

3. Isolation of the virus

3.1 Isolation of the virus and viral growth on pig's lung alveolar macrophages

A culture of pig's lung alveolar macrophages, previously prepared in DMEMs medium and FCS at 10%, was infected with a homogenate of a sample suspected of containing the causative agent of PRRS. The sample was composed, in one case, of a lung isolate from a stillborn piglet born to a sow that presented clinical signs of PRRS, whereas in other cases isolate was used from a piglet born alive but which had died after a few hours, progeny of a sow with PRRS symptoms, as well as an isolate from blood plasma. The homogenate was kept in contact with the macrophage culture at 37°C for 1 hour. Afterwards, the inoculum was removed and fresh DMEMs with 2% FCS and 1% PEG solution were added, buffering the culture with CO_2 and allowing to incubate at 37°C for several days during which the cytopathic effect (CPE) produced by the virus on the macrophages was observed under microscope: at 3-4 days post infection (dpi) CPE was 70-80% (giant and deformed cells appeared). The cultures were frozen at -80°C.

Simultaneously, a pig's lung alveolar macrophage culture free of PRRS was prepared to be used as negative control.

Subcultures were prepared with the isolated virus and it was observed that there was 100% CPE from the second dpi. The virus was frozen at -80°C for its posterior identification and characterization.

Similarly, the virus was isolated from blood plasma and other samples from stillborn piglets or living but weak piglets born to experimentally infected sows.

One of the isolated viruses, in particular the virus denominated **PRRS-CY-218-JPD-P5-6-91**, was isolated from a stillborn piglet's lung. It is capable of experimentally reproducing the disease, has the characteristics mentioned in Section 4 and was deposited at the European Collection of Animal Cell Cultures (ECACC), Salisbury (United kingdom), on June 29, 1993 with accession no. V93070108.

3.2 Viral growth in other cell systems

Infections with the isolated virus (Spanish strain) have been carried out in pig's lung alveolar macrophage and ST cell [Swine Testis continuous cell line) ATCC CRL 1745 ST] co-cultures, as a first step for the adapting of the virus to ST cells. After several serial passages (5-6) on the ST cell and macrophage co-cultures and cultures of ST alone, the virus infectious titres were of the order of 10^6 $TCID_{50}$/ml when macrophage-ST cell co-cultures were infected, and of the order of $10^{4.5}$ $TCID_{50}$/ml for the virus obtained in ST cells alone. [$TCID_{50}$ tissue culture infectious dose 50%].

Additionally, pig's lung alveolar macrophages can also be immortalized by fusing them with ST cells by means of hybridization.

Alternatively, pig's lung alveolar macrophages can be immortalized by fusing them with L-14 cell line (porcine peripheral blood B cells) ECACC no. 91012317, or with cell line Jag-1 (porcine trophoblast cell line) supplied by Dr Jag Ramsoondar

The fusion procedures are done following conventional techniques of use in this field.

Alternatively, viruses can be grown on ST cells or on any other porcine cell line in which have been introduced the genes coding for pig's lung alveolar macrophage membrane receptors for the PRRS virus.

The viruses produced with these cell systems are susceptible of being used in the formulation of both living as well as inactivated vaccines.

4 Identification and characterisation of the virus

4.1 Denomination and depositing of the virus

The virus isolated from a stillborn piglet's lung, denominated **PRRS-CY-218-JPD-P5-6-91**, was deposited at the European Collection of Animal Cell Cultures (ECACC), Salisbury (United Kingdom), on June 29, 1993 with accession no. V93070108. In the present description, occasionally, the virus is described without distinction as Spanish virus (SV) or Spanish strain.

4.2 Characteristics of the isolated virus

This virus (**PRRS-CY-218-JPD-P5-6-91**) presents the following characteristics:

a) the production of slight CPE on a continuous ST cell line

(ATCC CRL 1746 ST) (fetal swine testis) with average titre of $10^{4.5}$ $TCID_{50}$/ml and on pig's lung alveolar macrophages with average titres of $10^{5.5}$ $TCID_{50}$/ml.;

b) when it infects pig's lung alveolar macrophage and ST cell co-cultures, average titres of $10^{6.3}$ $TCID_{50}$/ml are obtained [which titres represent a logarithm unit (1 $\log_{10}$) higher than when only pig's lung alveolar macrophages are infected];

c) cytoplasmic replication;

d) production of cytoplasmic vacuolation;

e) lipid envelope;

f) 40-50 nm size;

g) no hemadsorption or hemagglutination observed with chicken, guinea pig, pig or human group O red blood cells;

h) loss of infectivity at acid pH (pH $\leq$5);

i) production of microscopic (interstitial pneumonia) and macroscopic lesions in 2-month old piglets;

j) production of adverse reproductive effects in pregnant sows with stillbirths, mummified fetuses and live but weak piglets;

k) cross-reaction with Lelystad reference serum (IPMA = Immuno peroxidase monolayer assay);

l) cross-reaction with sera from animals with clinical field infections (IPMA);

m) serum from sows infected with this virus cross-reacts with LV;

n) RNA polyadenylated genome of approximately 15000 bp in length (neutral agarose gel);

o) replication by means of a subgenomic RNA set present at end 3';

p) nucleotide sequence having 8 ORFs;

q) in a purified suspension subjected to electrophoresis in polyacrylamide gel and posterior transference by immunoblot were detected, by means of a specific serum, prepared in sows from our own laboratory and which cross-reacts with the Lelystad PRRS virus, 4 majority bands corresponding to protein of apparent molecular weights of 15000, 23000,

54000 and 66000 Daltons, which were not detected in negative controls (uninfected macrophages);

r) when ORFs 3 to 7 of this virus are compared with those of LV and/or TV, 95.5% homology is observed at nucleotide level (there are 114 different bp out of a total of 2599 bp) and 94.9% homology at amino acid level (51 different amino acids out of a total of 955); and

s) the virus belongs to the Arterivirus genus.

4.3 Techniques used for virus identification

4.3.1 Experimental reproduction of the disease in pregnant sows

German Landrace x Large White cross sows were used, originating from farms with systematic serological control against the viruses of Aujeszky's disease, foot-and-mouth disease, porcine parvovirosis, classic swine fever, swine influenza (types H1N1 and H3N2) and transmissible gastroenteritis. Additionally, the antibody titration test against the causative virus of PRRS was carried out. Between days 77 and 90 of gestation the animals were infected, with an isolate obtained on pig's lung alveolar macrophages in one case, intravenously (IV) and intranasally (IN), whereas in another case, only via intranaasal route (Example 2.1). Throughout the entire experiment feed intake, rectal temperature and clinical state of the animals were monitored. With the purpose of excluding the above mentioned agents, blood samples were taken from all the sows before infection. They proved to be seronegative to all the agents. Likewise, after the experimental infection, all the sows were still seronegative to all the said viruses and seropositive against PRRS (verified with the reference LV). The results obtained are shown in Table 1.

TABLE 1

TESTS CONDUCTED

INFE. AGENT	HAI (1)	HAI (2)	NPLA (3)	ELISA (4)	SN (5)	SN (6)	IPMA (7)
AD	ND	ND	ND	-	ND	ND	ND
FMD	ND	ND	ND	ND	-	ND	ND
PP	-	ND	ND	ND	ND	ND	ND
CSF	ND	ND	-	ND	ND	ND	ND
SF	ND	-	ND	ND	ND	ND	ND
TG	ND	ND	ND	ND	ND	-	ND
PPRS	ND	ND	ND	ND	ND	ND	+

KEY

AD	=	Aujeszky's disease.
FMD	=	Foot-and-mouth disease.
PP	=	Porcine parvovirosis.
CSF	=	Classic swine fever.
SF	=	Swine influenza (H1N1, H2N3).
TG	=	Transmissible Gastroenteritis.
PRRS	=	Porcine reproductive and respiratory syndrome.
HAI	=	Hemagglutination Peroxidase-linked Assay.
NPLA	=	Neutralizing Peroxidase-linked Assay.
ELISA	=	Enzyme-linked Immunosorbent Assay.
SN	=	Seroneutralization.
IPMA	=	Immuno peroxidase monolayer Assay.
(1)	=	Vannier et al., Rec. Med. Vet. 155 (2), 151-158 (1979).
(2)	=	Charley B., Doctoral Thesis, Alfort (1976).
(3)	=	Trepsta et al., Vet. Microb. 9, 113-120

(1984).

(4) = Elliot M., J. Rech. Porc., 20, 141-146.

(5) = Lucam F., "Diagnostic sero-immunologique des viroses humains et animals", F. Bricout; L. Joubert; J.M. Huraux (1977).

(6) = Jiménex et al., J. Virol., 60, 131-139 (1986).

(7) = Wensvoort et al., Vet. QUARTERLY, Vol. 13, n° 3 (July 1991).

(-) = Negative.

(+) = Positive.

ND = Not done.

4.3.2 Experimental reproduction of the disease in piglets

The objective of this experiment was to verify if the causative virus of reproductive alterations in sows was capable of producing in 2-month old piglets respiratory symptoms and macroscopic and microscopic lesions at lung level. To that end, a number of piglets were infected with virus (Spanish strain), IN route, which were then sacrificed on different post-infection days (Example 2.2).

The most relevant resulting data demonstrate that this virus produces at macroscopic level multiple foci of consolidation as well as interstitial pneumonia at microscopic level (Table 4). From the health point of view, no relevant clinical signs were observed.

4.3.3 Sensitivity to chloroform test

This test was conducted to determine if the isolated virus had lipid envelope. To that end the Feldman, H. and Wang, S., method was used, described in "A manual of basic virological techniques", Prentice-Hall Inc., New Jersey, 146-148 (1978). The results obtained demonstrate that the untreated virus was a titre of $10^{5.6}$ $TCID_{50}$/ml, whereas after treatment with chloroform the

titre is lower than $10^{1.3}$ $TCID_{50}$/ml, based on which it can be stated that the isolated virus has lipid envelope.

4.3.4 Sequencing of the viral genome

i) Purification of the virus
ii) Purification of the viral RNA
iii) cDNA synthesis
iv) Cloning and characterization of the cDNA clones
v) Sequencing and comparing of the sequences with those of LV

i) Purification of the virus

Virus replicated on pig's lung alveolar macrophages was clarified, and concentrated by filtration using MILLIPORE filters. Afterwards, the virus was subjected to centrifugation in 10% to 50% metrizamide gradient (SIGMA). Once centrifugation was completed, a band has obtained which was concentrated by centrifugation. With the purified virus, an electrophoresis in polyacrylamide gel was done and an immunoblot was developed with a specific serum, showing proteins whose apparent molecular weights were 15, 23, 54 and 66 K Daltons.

ii) Purification of the viral RNA

A technique was used for selection and purification of the RNA based on the fact that the RNA contains poly (A) sequence at the 3' end. For that purpose, a commercial kit (Pharmacia) was used which exclusively allowed the binding of the RNA poly (A) chain to a cellulose-oligo (dT) matrix and its posterior elution.

iii) cDNA synthesis

A commercial kit was used (Boehringer Mannheim), following the manufacturer's instructions. Briefly, the viral genomic RNA

was incubated in the presence of an oligo (dT), dATP, dCTP, dGTP, dTTP and reverse transcriptase.

iv) Cloning and characterization of the cDNA clones

The cDNA was cloned in a vector derived from pUC18 and a series of clones was obtained containing the complete sequence of nucleotides corresponding to ORFs 3 to 7.

v) Sequencing and comparing of the sequences with those of LV

v.a.) Sequencing

The region corresponding to ORFs 3-7 of the virus isolated at our laboratories has been sequenced completely. Figures 1 to 5 show the sequences of cDNA obtained as well as the sequences deduced from amino acids coded by each ORF. The total sequenced region length is of approximately 2599 nucleotides (nt).

As can be seen, ORF 3 has a length of about 798 nt and codes for a protein of 266 amino acid residues.

ORF 4 has a length of approximately 552 nt and codes for a protein of 183 amino acid residues. The beginning of this ORF 4 is located in the ATG codon located at about 540 bp from the initial ORF 3 ATG codon. ORF3 and ORF 4 share a sequence of about 246 nt.

ORF 5 has a length of about 606 nt and codes for a protein of 200 amino acid residues. The initial codon of this ORF 5 practically overlaps the ORF 4 end codon (they share the TG nucleotides, the ATG codon at the beginning of ORF 5 and the ORF 4 TGA end codon). The ORF 5 ATG initial codon is located about 1092 nt from the ORF 3 initial ATG codon.

ORF 6 has a length of about 522 nt and codes for a protein of 173 amino acid residues. This ORF 6 initial ATG codon is

located 8 nt upstream from the beginning of the ORF 5 termination TAG codon (at about 1682 nt approximately from the initial ORF 3 ATG codon).

ORF 7 has a length of some 387 nt and codes for a protein of 129 amino acid residues. This ORF 7 initial ATG codon is located 5 nt upstream from the beginning of the ORF 6 termination TAA codon (at about 2193 nt approximately from the ORF 3 initial ATG codon).

The proteins coded by ORFs 3 to 6 are membrane proteins, whereas the protein coded by ORF 7 is a nucleocapsid protein

v.b.) Comparison with LV

Comparing the cDNA sequences of ORFs 3-7 of LV with those of the virus isolated at our laboratories it is observed that:

i) at nucleotide level, out of the 2599 nucleotides compared 114 are different, which represents approximately 95.5% homology,

ii) at amino acid level, out of the 955 amino acids compared, there are 47 different ones, representing 94.9% homology approximately,

iii) of the 47 different amino acids there are 35 considered as non-conservative substitutions, of which there are:

- 12 in the product of the ORF 3 gene (Figure 6);
- 9 in the product of the ORF 4 gene (Figure 7);
- 10 in the product of the ORF 5 gene (Figure 8), although it is convenient to point out that the product of the LV ORF 5 gene contains one amino acid more than the product of the Spanish virus ORF 5, specifically amino acid 35 (Asn) of the LV ORF 5 product is not present in the product

expressed by the Spanish virus;

- 4 in the product of the ORF 6 gene (Figure 9); whereas,
- the product of the ORF 7 gene does not contain any non-conservative substitution (Figure 10),

iv) partial homology of each one of the products expressed by the different ORFs of the Spanish virus and LV is the 93.6% for the ORF 3 and ORF 5 products, 94.0% for the ORF 4 product, 96.6% for the ORF 6 product and 99.2% for the ORF 7 product.

As mentioned above, the changes in the amino acids may be connected to the higher pathogenicity of one strain in comparison with another, since the virus isolated at our laboratories (Spanish strain) is more pathogenic than other known PRRS viruses like, for example, the virus isolated in France (Example 8) and LV (Table 6 of PCT request no. WO 92/21375).

5 Vaccines

The invention provides a vaccine capable of preventing porcine reproductive and respiratory syndrome (PRRS). The vaccine has proved to be efficacious in preventing reproductive alterations in sows, such as stillborn piglets, mummified piglets or live but weak piglets, return to service and similar problems, produced by the virus causative of PRRS. Likewise, it has been verified that the vaccine induces cellular immunity in vaccinated animals.

The vaccine contains a suitable amount of PRRS viral antigen, Spanish strain, inactivated, as well as an adjuvant and a preservative.

Tests conducted with these vaccines have demonstrated the efficacy of the vaccine, as manifested by Examples 7 and 8. Additionally, the vaccine has demonstrated to be effective in

avoiding return to service, which appears in infected sows. Actually, the sows vaccinated with the vaccines resultant from this invention and infected with the causative virus of PRRS mated and became pregnant at the first post-partum ovulation and weaning of the piglets.

5.1 Components

5.1.a Antigenic phase

An active component, the vaccine contains inactivated PRRS viral antigen, Spanish strain, at a concentration higher or equal to $10^{5.5}$ $TCID_{50}$ per vaccinal dose. The inactivation can be done by chemical means that include treatment with ß-propiolactone or with other conventional inactivating agents such as ethylenimine or formaldehyde, or by physical means.

5.1.b Adjuvants

Although it is possible to use adjuvants of aluminum hydroxide type, Quil A or their mixtures, as well as oily adjuvants, for the formulation of the vaccine, it has been possible to verify that the best results are obtained when an oily adjuvant is used (Example 4). In particular, it has been verified that an oily adjuvant constituted by a mixture of Marcol 52, Simulsol 5100 and Montanide 888 affords very good results.

Marcol 52 is a low-density mineral oil manufactured by ESSO ESPAñOLA, S.A.; Simulsol 5100 is a polyethoxy oleate ether commercialized by SEPIC; and Montanide 888 is an anhydrous mannitol ether octadecenoate of high purity, commercialized by SEPIC.

It has been possible to verify that the adjuvant plays an essential part in the efficacy of the vaccine. Thus, a challenge test (Example 6) was conducted using sows vaccinated with two different vaccines, one sow with the oily adjuvant indicated

above (Ref. 1) and the other sow using the adjuvant Munokynin^{R} (Ref. 2). Although both vaccines produce seroconversion, it was verified in an experimental infection test that the sows vaccinated with vaccine Ref. 1 were protected against experimental infection with PRRS virus, whereas the other sows, those vaccinated with vaccine Ref. 2, were not protected in spite of the fact that at the time of challenge they had antibodies against the virus. This establishes that a suitable adjuvant can play a very important part in connection with the modulation and stimulation of the immune response, principally at cellular immunity level. Additionally, this aspect has been confirmed by means of the challenge test carried out using the Spanish strain of the PRRS virus, since the sows vaccinated and revaccinated with Ref. 1 vaccine did not present serological response at the time of infection but, nevertheless, they were protected (Example 7. Table 8).

However, it could also be possible that Ref. 2 vaccine (with Munokynin^{R}) might provoke cellular immunity. To that end, it would be necessary to add substances that potentiate cell response (CRP) that is substances that potentiate helper T-cell subpopulations (Th_1 and Th_2), such as IL-1 (interleukin-1), IL-2, IL-4, IL-5, IL-6, IL-12, g-IFN (gamma interferon), cellular necrosis factor and similar substances. Evidently, it would also be possible to add these CRP substances to vaccines with oily adjuvant, in which case their cell immune effect would be potentiated.

Other types of adjuvant may also be used that modulate and immunostimulate cell response, such as MDP (muramyl dipeptide), ISCOM (Immuno Stimulant Complex) or liposomes.

5.1.c Preservatives

Any of the preservatives habitually used in the formulation of vaccines may be used. One of these is Thimerosal [sodium salt of (2-carboxy-phenylthio) ethyl-mercury] (ALDRICH).

5.2 Method for the preparation of the vaccine

The vaccines resultant from this invention can be obtained by the mixing of an antigenic phase containing inactivated viral antigen and another phase, as adjuvant, which may or may not be oily, depending on the adjuvant chosen. Optionally, CRP substances could be added to any of the two phases. When the adjuvant is oily, an emulsion is formed which, in a particular and preferable case (when the adjuvant is a mixture of Marcol 52, Simulsol 5100 and Montanide 888), is a double w/o/w (water/oil/water) emulsion.

5.3 Vaccine control tests

In addition to the conventional tests the vaccine must pass before its administration, for (i) purity (against bacteria, fungi, mycoplasmas and foreign viruses) (ii) identification, (iii) safety, (iv) potency and (v) physico-chemical controls, a number of field trials (Example 5.b) have been carried out in relation to the safety and efficacy of the vaccine in a total of 5 farms, of which 508 sows were vaccinated and revaccinated with one of the vaccines resultant of this invention, whereas the remaining 472 sows were not vaccinated and were kept as control sows, making it possible to observe that the vaccine is safe and at the same time effective, for in some of the farms the natural disease PRRS was detected in unvaccinated animals after the process of vaccination.

5.4 Posology and instructions for the administration of the vaccine

It has been possible to verify that one dose of 2 ml of oily vaccine with a concentration of inactivated viral antigen equal to or higher than $10^{5.5}$ $TCID_{50}$, administered via deep intramuscular route, is capable of protecting a very high percentage of vaccinated animals against PRRS.

The following vaccination programm is advisable:

* First vaccination: vaccinate all breeding animals (sows and boars) and revaccinate 21 days later. Afterwards, administer one dose during every lactation (sows), and every 6 months (boars).

* Posterior vaccinations
  - Animals intended for reproduction: The first vaccination should be at least 6 months of age, revaccinating 21 days later.
  - Sows: It is advisable to vaccinate during the period of lactation, if possible 15 days before mating.
  - Boars: Vaccinate twice a year (every 6 months).

Alternatively, if no CRP substance has been included in the vaccine, these substances may be injected at a site different to the site of inoculation but simultaneously.

6. Polyvalent vaccines

By means of an additional objective attained from this invention, combinations are provided of the different porcine pathogens containing in addition to inactivated viral PRRS antigen (Spanish strain) one or more of the pathogens listed below, in order to enable the preparation of bi- or multivalent vaccines.

This way bi- or multivalent vaccines may be prepared containing inactivated viral PRRS antigen, and one or more of the following pathogens: Actinobacillus pleuropneumoniae, Haemophilus parasuis, Porcine Parvovirus, Leptospira, Escherichia coli, Erysipelothrix rhusiopathiae, Pasteurella multocida, Bordetella bronchiseptica, Porcine respiratory coronavirus, Rotavirus or against the pathogens causative of Aujeszky's disease, swine influenza or transmissible gastroenteritis.

EXAMPLES

Example 1 Isolation of the virus

1.A Preparation of the samples

From the lung of a stillborn piglet, progeny of a sow with the classic PRRS symptoms [the sow was free of antibodies against Aujeszky's disease, porcine parvovirosis, foot-and-mouth disease, classic swine fever, swine influenza (types H1N1 and H3N2) and transmissible gastroenteritis], a 10% suspension was made in DMEM culture medium, supplemented with a solution of antibiotics (PEG) composed of 1000 IU/ml of penicillin, 1 mg/ml of streptomycin and 0.5 mg/ml of gentamicin, at a ratio lung:DMEM solution of 1:10 (W/V). The suspension produced was homogenized and left to stand for 1 hour at room temperature (20-22°C). The homogenate was frozen and thawed twice, centrifuged and the supernatant obtained stored at -70°C, to be used in the infection of pig's lung alveolar macrophages. Similarly, samples were prepared from the lung of a piglet born alive but which died within a few hours. Additionally, blood was extracted and mixed with and without anticoagulant and used for virus isolation (from blood plasma) and to obtain serum, respectively.

1.B Obtainment of pig's lung alveolar macrophages

Alveolar macrophages were obtained from the lungs of pig's seronegative to Aujeszky's disease, porcine parvovirosis, foot-and-mouth disease, classic swine fever, swine influenza (types H1N1 and H3N2) and transmissible gastroenteritis. The age of the pigs used ranged between 7 and 8 weeks. Prior to the extraction of the lungs, the animals were anaesthetized with phenobarbital sodium and then sacrificed. Immediately, the lungs together with the trachea were extracted after ligating it below the epiglottis. The extracted lung was washed externally with physiological saline solution, and in successive washings 50 ml

of PBS supplemented with 2% of PEG solution of antibiotics were introduced until a total of 500 ml of PBS had been introduced. The cells obtained from these washings were centrifuged for 10 minutes at 300g. This step of washing centrifugation was repeated two more times. The cells obtained were washed with PBS and PEG solution of antibiotics and resuspended in DMEMs medium [DMEM supplemented with non-essential amino acids (GIBCO), 1% sodium pyruvate 1mM and 1% of glutamine 2mM], 10% fetal calf serum (FCS) and PEG solution of antibiotics at 1%. The cell count was done in Newbauer chambers and to that end 1/10 dilution of the macrophage suspension was prepared by adding 0.4 ml of DMEMs and 0.5 ml of trypan blue solution to 0.1 ml of macrophage suspension. A number of cells ranging between 1 and 1.2 x 10^9 was obtained.

1.C Isolation of the virus

A culture flask of 25 cm^2 surface containing a culture of pig's lung alveolar macrophages previously prepared (3 x 10^6 cells/ml) in DMEMs medium and 10% FCS was infected with 1 ml of the homogenate of a sample originating from the lung of a stillborn piglet (Example 1.A). The homogenate was left in contact with the macrophage culture for 1 hour, at 37°C, buffered with CO_2 at pH7.0-7.4, and incubated at 37°C for several days during which the CPE produced by the virus on the macrophages was observed. At 3-4 dpi, CPE was observed to be 70-80%, for which reason the cultures were frozen at -80°C.

Simultaneously, a culture was prepared of uninfected pig's lung alveolar macrophages, used as negative control.

Subcultures with the isolated virus were done in which it was observed that CPE starting from the second dpi was 100%. The virus was frozen at -80°C for its posterior identification and characterisation. After the fourth passage in macrophages the corresponding titrations in 96-well microplaques were done, obtaining an average titre of $10^{5.6}$ $TCID_{50}$/ml in accordance with

the Reed & Muench method [Am. J. Hyg., 27: 493-497 (1938)].

A sample of isolated virus (Spanish strain) denominated PRRS-CY-218-JPD-P5-6-91, isolated from a stillborn piglet's lung, is capable of experimentally reproducing the disease and was deposited at the ECACC on July 1, 1993, under corresponding accession no. V93070108, under the terms of the Budapest Treaty.

In a similar way, the virus was isolated in live and stillborn piglets, progeny of sows infected experimentally.

Example 2 Identification and characterisation of the virus

Example 2.1 Experimental reproduction of the disease in pregnant sows

Twelve sows, German Landrace X Large White cross were used, originating from farms with systematic serological control against the viruses of Aujeszky's disease, foot-and-mouth disease, porcine parvovirosis, classic swine fever, swine influenza (types H1N1 and H3N2) and transmissible gastroenteritis. Additionally, the antibody evaluation test against the causative virus of PRRS was conducted.

The sows were moved to the safety stables of the research center one week before infection and placed in separate stables. Between days 77 and 90 of gestation, the sows identified with numbers 53, 76, 8, 62, 91, 93 and 19 were infected with 5 ml intravenous route (IV) and with 5 ml intranasal route (IN) of PRRS virus, Spanish strain, isolated on pig's lung alveolar macrophages identified as PRRS-CY-218-JPD-P5-6-91, from a fourth passage on macrophages filtered through a 200 nm filter and with a titre of $10^{5.6}$ $TCID_{50}$/ml. The 5 remaining sows (nos. 14, 40, 13 30 and 85) were inoculated between days 65 and 85 of gestation with 5 ml via IN (only) of the virus.

During the experiment feed intake, rectal temperature and the clinical state of the animals were monitored daily, as well as reproductive alterations (premature parturitions, delayed parturitions, live but weak piglets, stillborn piglets, mummified, and healthy piglets.).

In order to proceed with the exclusion of the above mentioned pathogens, blood samples were taken from the sows pre and post infection, with the result that the animals were seronegative to the pathogens prior to and after infection and seropositive against PRRS after infection (see Table 1, section 4.3.1).

The reproductive results appear in Tables 2 and 3. As shown in Table 2, of the 93 piglets farrowed, 15 were mummified, 35 stillborn, 22 were born alive but died on the third day and 21 survived 7 days of life.

Some sows manifested inappetence for 2-4 days, on days 6 and 8 post infection, whereas other sows manifested inappetence on the second day post infection. Hyperthermia was not observed in any case. In 4 sows (nos. 8, 62, 92 and 93) farrowing was 1 to 6 days premature, whereas in the other 3 sows farrowing was delayed 1 or 2 days.

Of the piglets born alive, 1 or 2 per litter manifested oedema in the eyes. The weak piglets manifested incoordination, paresis of the hind quarter, bristling hair and myoclonia. In the necropsy effected on some of the stillborn and weak piglets the presence of abundant clear liquid was observed in the thoracic cavity. Healthy piglets born to infected mothers sacrificed at 8-12 days of life manifested gray foci of consolidation. Under microscopic observation the most significant change was a slight multifoci interstitial pneumonia with enlargement of alveolar septi because of the infiltration of mononuclear cells. These lesions appeared in all the animals analyzed in the experiment.

As seen in Table 3, out of 65 piglets farrowed, 36 were stillborn, 26 were born alive but weak, dying on the second day of life, and 3 survived the first week of life. One sow farrowed 12 days prematurely (no. 40) whereas the others farrowed 1 or 2 days prematurely. The clinical signs of the piglets born weak are similar to that obtained via IN+IV. Interstitial pneumonia was observed also. The infected sows did not manifest inappetence or hyperthermia.

The most relevant difference between the two systems of infection is that via IN + IV mummified piglets were observed and that 1 to 2 of the piglets born alive in each litter manifested edema around the eyes.

In view of the results obtained, it can be concluded by stating that the model for experimental infection in sows, at about 80 days of gestation via both routes of infection (IN and IV) with the virus isolated (Spanish strain) from animals infected naturally, reproduces the disease PRRS in pregnant sows and provokes a high proportion of mummified fetuses, stillborn piglets and live but weak piglets very similarly to the proportion observed in acute natural infection outbreaks. It is advisable to infect artificially via IN route for reason that this is the natural route of infection in the field and therefore the most appropriate way to evaluate the efficacy of the vaccine.

By means of this experiment it has also been possible to set up a model for experimental infection in pregnant sows that allows for the verification of the efficacy of the vaccine.

TABLE 2

IN + IV

							H		
A	B	C	D	E	F	G	h1	h2	I
53	77	115	9	3	2	0	-	4	4/4
76	77	116	11	2	4	1	-	4	NT
8	77	110	12	2	4	1	-	5	5/5
62	80	113	16	-	3	6	4	3	3/3
91	90	109	14	-	1	8	1	4	NT
93	88	110	17	4	2	11	-	-	NT
19	88	116	14	4	1	8	-	1	NT
			93	15	17	35	5	21	

TABLE 3

IN

							H		
A	B	C	D	E	F	G	h1	h2	I
14	65	111	15	-	-	12	-	3	NT
40	82	102	12	-	-	12	-	-	NT
13	80	113	12	-	10	2	-	-	NT
30	80	113	15	-	12	3	-	-	NT
85	85	112	11	-	4	7	-	-	NT
			65	-	26	36	-	3	

A: Sow
B: Time of infection (days of gestation)
C: Farrowing (days of gestation)
D: Total piglets
E: Mummified piglets
F: Weak piglets dead by 48 h.
G: Stillborn piglets
H: Piglets apparently in good health.
h1: Dead between days 2 and 7.
h2: Living after one week.
I: Interstitial pneumonia.
NT: Not tested.

Example 2.2 Experimental reproduction of the disease in piglets

This experiment was designed with the purpose of verifying that the isolated virus (Spanish strain), which produces reproductive alterations in sows, is capable of producing clinical signs as well as macroscopic and microscopic lesions in the lungs of 2-month-old piglets. To that end, 10 piglets were infected via IN route with 5 ml of virus, Spanish strain, with a titre of $10^{5.6}$ $TCID_{50}$/ml and 6 other piglets were left as controls (all of them originated from 2 litters). The animals were sacrificed on days 3, 7, 8, 9 and 11 post infection. The results obtained are shown in Table 4.

TABLE 4

No.	I/C	DPI	Antibodies	I.P.	V.I.
2	I	3	-	2+	NT
12	I	3	-	2+	NT
11	C	3	-	NL	NT
1	I	7	1:80	4+	+
14	I	7	1:80	2+	+
13	C	7	-	NL	-
10	I	8	1:160	4+	+
15	I	8	1:160	2+	+
8	C	8	-	NL	-
17	C	8	-	NL	-
4	I	9	1:160	4+	+
5	C	9	-	NL	-
20	I	11	1:160	4+	+
18	I	11	1:320	3+	+
9	I	11	1:320	3+	-
6	C	11	-	NL	-

No. : Pig number
I/C : Infected (I) or Control (C)
DPI : Days post infection
I.P. : Interstitial pneumonia
V.I. : Virus isolation
2+ : Slight interstitial pneumonia
3+ : Intermediate interstitial pneumonia
4+ : Serious interstitial pneumonia
NT : Not tested
NL : No lesions

During the 11 days of the experiment, no clinical respiratory signs or hyperthermia were observed, although there was loss of weight in the infected animals in comparison with uninfected animals. Under microscopic observations, the most relevant aspect was the presence of multiple foci of consolidation in the lungs, congested lymph nodes in the mandible and some intestinal haemorrhages. At microscopic level interstitial pneumonia was observed.

Virus was isolated from the solutions obtained from the washings of infected animals' lungs on a fresh macrophage culture, but virus was not isolated from the control animals, in which seroconversion was not observed nor macroscopic or microscopic lesions at lung level.

When cell counts were done from the washings of infected animals' lungs, 30% dead cells (macrophages) was observed, which may constitute a factor of importance in secondary infections because of the destruction of a key immunologic defense element (macrophages).

The absence of respiratory signs may be due to the fact that the experimental infections were carried out in stables with continuous disinfection treatments and, therefore bacterial concentration was much smaller when compared with that existing

in herds under field conditions.

Example 2.3 Sensitivity of chloroform test

The Feldman, H. and Wang, S. method (Section 4.3.3) was used resulting in the knowledge that the isolated virus has lipid envelope since there is a drop in titre of 4 $\log_{10}$ from the control cultures of those treated with chloroform.

Example 2.4 Sequencing of the viral genome

i) Purification of the virus

The virus, replicated on pig's lung alveolar macrophages was purified by filtration and centrifugation in 10% to 50% metrizamide gradient (SIGMA), resulting in a band which was centrifuged again as mentioned in section 4.3.4. With the purified virus was conducted an electrophoresis in polyacrylamide gel, and an immunoblot developed with a specific serum, showing proteins with apparent molecular weights of 15, 23, 54 and 66 K Daltons.

ii) Purification of the viral RNA

The viral RNA was purified using a commercial kit (PHARMACIA) that enables the binding of the RNA poly (A) tail to cellulose-oligo (dT) matrix and its posterior elution.

iii) cDNA synthesis

A commercial kit was used (BOEHRINGER MANNHEIM) (section 4.3.4 iii).

iv) Cloning and characterization of the cDNA clones

The cDNA was cloned in a vector derived from pUC18 and a

series of clones was obtained containing the complete nucleotide sequence corresponding to ORFs 3 to 7.

v) Sequencing and comparing of the sequences with those of LV

The results of the sequencing of the cDNA of the virus isolated at our laboratories (ORFs 3-7) as well as the comparison between that sequence and the LV sequence are mentioned in section 4.3.4.v, where it can be seen that, at amino acid level, there is approximately 94.9% degree of homology and a total of 47 different amino acids of which 35 correspond to non-conservative substitutions. These differences at amino acid level may be responsible for the different pathogenicities that exist between the various PRRS virus strains isolated.

Example 3 Formulation of a vaccine

A vaccine, capable of protecting against PRRS, is prepared in emulsion form following the procedure described below.

A pig's lung alveolar macrophage culture is infected with MOI (multiplicity order infection) of 0.001 and incubated at 37°C for 24 hours, at the end of which the culture medium is substituted by infection medium (DMEM supplemented with 2% FCS). The culture is incubated for 4 days at 37°C until 70-80% CPE is observed. Once this period is completed an IPMA test is conducted in order to confirm identification. The virus is collected by vacuum aspiration and frozen at -80°C.

The viral suspension destined to the formulation of vaccine should have $10^{5.5}$ $TCID_{50}$/ml minimum titre (prior to its inactivation) and should not be contaminated by bacteria, fungi, mycoplasmas or other viruses. In the case that the titre may be lower, it should be adjusted by concentrating the antigen.

For the inactivation of the viral suspension, 2% ß-propiolactone solution is added and stirred at 4°C for one night, maintaining the pH at 7.4 by adding 0.5M NaOH. Once the inactivation period is completed, the viral suspension is maintained at 37°C for 1 hour.

The following is then prepared:

a) an antigenic phase of the viral antigen inactivated with minimum concentration of $10^{5.5}$ $TCID_{50}$/dose and the preserver; and

b) an oily phase composed of Marcol 52, Simulsol 1500 and Montanide 888.

The aqueous phase, maintained in stirring, is added slowly to the reactor containing the oily phase maintained also in stirring. Once the aqueous phase has been added completely stirring is continued for 10 minutes.

In a particular, preferred case vaccines have been prepared capable of preventing PRRS, comprising per dose of 2 ml:

a) 53% of an antigenic phase, containing:
   i) PRRS viral antigen in DMEM culture medium, Spanish strain inactivated with ß-propiolactone, at minimum concentration of ..... $10^{5.5}$ $TCID_{50}$ and
   ii) thimerosal ..... 0.01%; and

b) 47% of an oily phase, containing:
   i) Marcol 52 ..... 790.0 mg
   ii) Simulsol 5100 ..... 70.0 mg
   iii) Montanide 888 ..... 80.0 mg

The oily Phase/Aqueous Phase ration is a weight/volume (W/V) ratio. This vaccine has been denominated **MSD Ref. 1.** The obtained vaccine is subjected to the corresponding control tests prior to use.

Another vaccine was similarly prepared using Munokynin^{R}

(aluminium hydroxide and Quil A, supplied by AMERICAN CYANAMID) as adjuvant, maintaining the same amount of inactivated virus. This vaccine has been denominated **MSD Ref. 2.**

Example 4 Evaluation of adjuvant

A field trial was conducted with a total of 128 sows out of which 49 were vaccinated with one dose of the vaccine denominated MSD Ref. 1, 50 sows were vaccinated with one dose of the vaccine denominated MSD Ref. 2 (Munokynin^{R}) and the remaining 29 were not vaccinated and were kept as controls. After 22 days, the sows were revaccinated with one dose of the corresponding vaccine.

The following parameters were evaluated.

1) Serological response by means of IPMA determination at the following times:
   - T_o : vaccination and bleeding
   - T_{22} : revaccination and bleeding
   - T_{51} : bleeding at 50 days post vaccination
2) General type reactions (appetence for feed, hyperthermia, etc.).

The results obtained are shown in Tables 5 and 6 which reflect the percentage of sows with positive serological reaction (Table 5) and the arithmetic mean of the serological titres reached (Table 6).

TABLE 5

% OF ANIMALS WITH SEROLOGICAL REACTION (+)

	T_o	T_{22}	T_{51}
MSD Ref. 1	-	59%	100%
MSD Ref. 2	-	40%	87%
Controls	-	-	-

TABLE 6

ARITHMETIC MEAN OF THE SEROLOGICAL TITRES

	T_0	T_{22}	T_{51}
MSD Ref. 1	-	58	200
MSD Ref. 2	-	37	133
Controls	-	-	-

No significant local or general type reactions were observed. It can be affirmed, based on the results obtained, that positive seroconversion is produced with both vaccines, although somewhat higher when the vaccine **MSD REF. 1** (oily adjuvant) is used. At revaccination, a higher seroconversion percentage is observed and the arithmetic mean of the titres obtained is higher in animals vaccinated with **MSD Ref. 1**.

Example 5 Safety in sows

Example 5.A At laboratory level

Example 5.A.1 Primiparous sows

Eighteen primiparous sows were chosen (German Landrace x Large White cross) from the porcine production farm and were distributed in two stables at the rate of 9 sows per stable, so that sows vaccinated with the same vaccine (**MSD Ref. 1** or **MSD Ref. 2**) obtained in Example 3 above were housed in the same stable.

Nine sows were vaccinated via deep IM route with one dose of 2 ml vaccine **MSD Ref. 1** containing $10^{5.5}$ $TCID_{50}$/dose inactivated virus titre, and were revaccinated with another dose of the same

titre 20 days later. The other 9 sows were vaccinated and revaccinated on the same days with the vaccine **MSD Ref. 2** (2 ml doses, $10^{5.5}$ $TCID_{50}$/dose inactivated virus titre).

During the first 5 days post inoculation the following observations were done:

a) Local reaction

Consisting of macroscopic observation of the site of inoculation and palpation, noting down the degree of inflammation in comparison with objects of known size.

b) General reaction

Consisting of macroscopic observation of the animals and verification of their appetence for feed. In negative case, rectal temperature is checked every 12 hours until hyperthermia or any other unfavourable signs have disappeared.

The obtained results reflect that there was a slight inflammatory reaction in some sows, prominent at the site of inoculation, disappearing in every case within a few days; no purulent formations were observed. Only one of the sows refused to ingest the totality of the feed in the first post-inoculation feeding, but feed ingestion was normal at the following feeding so that it was not necessary to take rectal temperature. No substantial differences in the response to the different tested vaccines were detected, based on which it can be affirmed that both vaccines are safe.

Example 5.A.2 Pregnant sows

Seven pregnant sows (German Landrace x Large White cross) from a porcine production farm were chosen at random: 6 primiparous sows of about 9 months of age and 1 multiparous sow, 3 years and 7 months old.

The vaccine denominated as **MSD Ref. 1** was used exclusively. The sows were vaccinated via deep IM route with a dose of 2 ml of vaccine that contained inactivated virus titre of $10^{5.5}$

$TCID_{50}$/ml, and 15 days later they were revaccinated with one dose of the same titre.

During the first 5 days post inoculation the following observations were done:

a) Local reactions

Consisting of macroscopic observations of the site of inoculation and palpation taking note of the degree of inflammation in comparison with objects of well-known size.

b) Inappetence

Consisting of macroscopic observation of the animals and checking for loss of appetence for feed.

c) Rectal temperature

Measuring of the rectal temperature at 24 hours post vaccination and 24 hours post revaccination.

The results obtained reflect that there was a slight local reaction in two of the animals which was not serious because of its small size, disappearing in a few days. Inappetence or hyperthermia were not observed in any case. Based on this, it can be affirmed that the vaccine is safe.

Example 5.B Safety and efficacy field trial

This experiment was carried out in the 5 farms listed below. A variable number of sows from each farm was vaccinated via deep IM with one dose of 2 ml of the vaccine **MSD Ref. 1** containing a titre of $10^{5.5}$ $TCID_{50}$/dose of inactivated virus, and revaccinated 21 days later with another dose of same titre, whereas the other sows were not vaccinated and were used as controls:

FARM	VACCINATED	CONTROL	TOTAL
I	19	11	30
II	46	34	80
III	153	147	300
IV	127	123	250
V	163	157	320
TL	508	472	980

TL = Total
I : Farm "RAMON DEL QUINTA" (Banyoles)
II : Farm "CAL SABATER" (Orriols)
III : Farm "E. CANELA" (Preixana)
IV : Farm "R. CUNILLERA" (L'Albi)
V : Farm "INVERSORS PICBER" (Bellpuig)

It has been possible to verify that the vaccine is safe after the observation of general and local reactions. Local reactions were only observed in 1% of the animals. In connection with the productive parameters observed in the above-mentioned farms, no variations were observed when compared with their clinical histories.

Regarding serological response, some farms have seroconversion to the vaccine while, in others, the response is negative. This is not indicative of low level of protection since in the experimental infection tests in the laboratory, seronegative animals resist experimental infection (Examples 7 and 8).

In connection with the transmission of maternal immunity from vaccinated animals to their progeny, there is a big drop in antibody titres at one month of age.

In vaccinated and revaccinated sows that are serologically positive there is a big drop in antibody titres at 2 months from revaccination.

Example 6 Verification of cell immunity

Five pregnant sows (German Landrace x Large White cross) from a porcine production farm were used. The animals were moved to the research center safety stables.

Two sows were chosen at random and were vaccinated with the vaccine denominated **MSD Ref. 1**. Another sow was vaccinated with

the vaccine denominated **MSD Ref. 2**. The 2 remaining sows were not vaccinated.

The sows were vaccinated deep IM route with one dose of 2 ml of vaccine **MSD Ref. 1** or vaccine **MSD Ref. 2** containing inactivated virus titre $10^{5.5}$ $TCID_{50}$, and 20 days later the sows were vaccinated with another dose of the same titre.

Afterwards, between days 77 and 90 of pregnancy all the sows were infected via IN route with 5ml of the virus **PRRS-CY-218-JPD-P5-6-91** with titre of $10^{5.8}$ $TCID_{50}$/ml. At the time of infection, it was verified that all the vaccinated sows presented antibodies against the causative virus of PRRS (positive serology). Table 7 shows the reproductive results obtained as a whole:

TABLE 7

(A)	(B)	(C)	(D)	(E)	(F)	(G)
2	MSD Ref. 1	23	20	--	20	3
1	MSD Ref. 2	12	--	6	--	6
2	--	24	--	7	--	17

(A) : No. of sows
(B) : Vaccine used
(C) : Total number of piglets
(D) : No. of piglets born alive in good health
(E) : No. of piglets born alive but weak
(F) : No. of piglets alive after the 1st week
(G) : No. of stillborn piglets

The results obtained demonstrate that the sows vaccinated with vaccine **MSD Ref. 1** (oily adjuvant) resist infection better than the sows vaccinated with vaccine **MSD Ref. 2** (aqueous adjuvant), which could mean that the adjuvant plays an important part in the establishment of cell immunity.

Example 7 Efficacy in pregnant sows

Eleven breeding sows were used (German Landrace x Large White cross) from a porcine production farm. The animals were moved to the research center safety stables.

Three sows were chosen at random (sows no. 57, 63 and 74) and were vaccinated with he vaccine denominated **MSD Ref. 1.** Three sows (no. 15, 18 and 23) were vaccinated with the vaccine denominated **MSD Ref. 2**, and the remaining 5 sows (no. 14, 40, 13, 30 and 85) were not vaccinated.

The sows were vaccinated via deep IM route with one dose of 2 ml of the vaccine denominated **MSD Ref. 1** or the vaccine **MSD Ref. 2**, containing inactivated virus titre of $10^{5.5}$ $TCID_{50}$/dose, and revaccinated with another dose of the same titre 20 days later. Local and general reactions were observed.

Serological response in the animals were verified by means of the IPMA test in accordance with the following program:

T_0 : Bleeding and vaccination
T_{20} : Bleeding and revaccination
T_{42} : Bleeding
T_{78} : Bleeding and experimental infection
T_8 : Post experimental infection bleeding
T_{25} : Post experimental infection bleeding
T_{50} : Post experimental infection bleeding

Experimental infection was carried out in the research center safety stables. All the animals were infected at the rate of 5 ml of **PRRS-CY-218-JPD-P5-6-91** virulent virus with titre of $10^{5.8}$ $TCID_{50}$/ml via IN route. Serological response was noted down as well as the number of piglets born alive and stillborn to each sow. Pre and post colostrum bleedings were done in the piglets, and lung and brain samples were taken from the stillborn piglets and from the piglets sacrificed on different days for isolation

of virus and histologic cuts.

The results obtained are shown on Tables 8-10 below:

TABLE 8

Serological results

No.	T_0	T_{20}	T_{42}	T_{78}	T_8	T_{25}	T_{50}
15	--	--	1/160	1/160	1/640	1/1280	NT
18	--	--	1/80	1/80	1/320	1/640	1/320
23	--	1/160	1/160	NP	NP	NP	NP
57	--	1/160	1/160	1/320	1/320	1/1280	1/160
63	--	1/80	1/80	1/160	1/640	1/1280	1/160
74	--	1/80	NT	1/160	1/640	1/2560	NT
c14	--	--	--	--	1/1280	1/2560	NT
c40	--	--	--	--	NT	1/1280	NT
c13	--	--	--	--	NT	1/320	NT
c30	--	--	--	--	NT	1/320	NT
c85	--	--	--	--	NT	1/320	NT

NT : Not tested

NP : Not pregnant

TABLE 9

Results of farrowing

NO.	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)
15*	83	112	13	--	--	--	--	--	--	--
18	78	111	--	11	8	--	3	--	--	2
23*										
57	63	108	--	13	7	--	6	--	--	--
63	79	114	--	13	8	1	2	2	--	2
74*	83	112	6	--	--	--	--	--	--	--
c14	65	111	--	15	--	--	3	12	--	--
c40	82	102	--	12	--	--	--	12	--	--
c13	80	113	--	12	--	10	--	2	--	--
c30	80	113	--	15	--	12	--	3	--	11
c85	85	113	--	11	--	4	--	7	--	4

[a] : Not pregnant

* : These sows died because of excessively high environmental temperature. Cesarean was performed at 112 days of gestation.

(A) : Date of infection (days of gestation)
(B) : Farrowing (days of gestation)
(C) : Total number of piglets (cesarean)
(D) : Total number of piglets (born)
(E) : Piglets born in normal health
(F) : Piglets born weak
(G) : Live piglets born splay-legged
(H) : Stillborn piglets (dead)
(I) : Stillborn piglets (mummified)
(J) : Piglets dead by the first week

TABLE 10

Serology of the piglets

A. Vaccinated sows

1	2	3	4	5
15[a])		NT	NT	-
			3 Days	10 Days
18	1	-	NT	NT	-
	2	-	NT	NT	-
	7	NT	1/640	1/160	-
	8	NT	1/2560	1/320	-
	9	NT	1/1280	1/640	-
	10	NT	1/1280	1/320	-
	11	NT	1/1280 - 1/2560	1/640	-
	12	NT	1/2560	1/640	-
	13	NT	≥1/2560	1/160	-
	14	NT	1/1280	1/160	-
	15	NT	1/2560	1/160	-
23	NP	NP	NP	NP
			4 Days	8 Days
57	1	-	1/320 - 1/640	1/320	-
	2	-	1/320 - 1/640	1/160 - 1/320	-
	3	-	1/640	NT	-
	4	-	1/320 - 1/640	NT	-
	5	-	1/640	NT	-

			1 Day	8 Days	
63	1	NT	≥1/2560	1/640	-
	2	NT	≥1/2560	1/320	-
	3	NT	1/2560	1/320 - 1/640	-
	4	NT	1/2560	1/640 - 1/1280	-
	5	-	1/1280	1/320	-
	6	-	1/2560	1/640	-
	7	NT	1/640 - 1/1280	1/320	-
	8	NT	1/1280	NT	-
74[a])			NT		-

B. Control sows (not vaccinated)

1	2	3	4				5
			12 H.	38 H.	5 days	9 Days	
c14	1	NT	≥1/2560	≥1/2560	1/1280	1/640	-
	2	-	≥1/2560	≥1/2560	1/1280	1/1280	-
	3	≥1/2560	≥1/2560	1/1280	1/1280	1/1280	-
c40		-		NT			

			9 Days	
c13	1	-	1/160	+
	2	-	1/160	-
	3	-	1/320	-
	4	-	1/320	-
c30		-	NT	NT
c85		NT	NT	NT

Key:

[a]) : These sows died because of excessively high environmental temperature. Cesarean was performed at 112 days of gestation.

1 : Number assigned to each animal.

2 : Piglets (the number assigned to each piglet corresponds to the order of birth)

3 : Pre-colostrum serology

4 : Post-colostrum serology

5 : Isolation of the virus

NT : Not tested
\- : Negative
\+ : Positive
H : Hours
NP : Not pregnant

It is evident from the results obtained that there is positive seroconversion against the causative virus of PRRS. Additionally, there is a satisfactory behaviour against experimental infection, in comparison with the control animals in which death was produced in the majority of the fetuses. Consequently, it can be affirmed that this vaccination is an efficacious measure for the prevention of PRRS.

Example 8 Efficacy of the vaccine against experimental infections with another agent causative of PRRS (cross-protection)

This experiment was designed for the verification of the efficacy of the vaccine identified as **MSD Ref. 1** in an experimental infection test using 2 pathogenic strains of the causative virus of PRRS.

The pathogenic PRRS strains used were:

i) Spanish strain, PRRS-CY-218-JPD-P5-6-91; and

ii) French strain, SDRP II 8B, provided by Dr. E. Albina of "Laboratoire Central de Recherches Avicole et Porcine", Ploufragan, France

The vaccine used was the vaccine denominated **MSD Ref. 1**, of which the formula is given in Example 5.

Thirty sows seronegative to the causative viruses of PRRS were used, at reproductive cycle not comprised between 10 days prior to nor 10 days posterior to mating, nor 10 days prior to nor 10 days posterior to farrowing.

Four groups of animals were formed:

A: 10 sows vaccinated and revaccinated via IM route and infected via IN route with Spanish strain PRRS-CY-218-JPD-P5-6-91 of the virus.

B: 5 sows not subjected to any vaccination and infected via IN route with Spanish strain PRRS-CY-218-JPD-P5-6-91.

C: 10 sows vaccinated and revaccinated via IM route and infected via IN route with French strain SDRP II 8B;

D: 5 sows not subjected to any vaccination and infected via IN route with French strain SDRP II 8B.

Experiment carried out

Twenty sows of the Groups A and C mentioned above were vaccinated with one dose of 2 ml of the vaccine denominated **MSD Ref. 1** via deep IM route and revaccinated 21 days later with the same dose of vaccine.

Afterwards, between days 70 and 80 of gestation, all the sows were infected experimentally with:

Groups A and B: 5 ml of virus PRRS-CY-218-JPD-P5-6-91 with titre of $10^{5.8}$ $TCID_{50}$/ml via IN route; and

Groups C and D: 5 ml of virus SDRP II 8B with titre of $10^{5.6}$ $TCID_{50}$/ml via IN route.

Parameters evaluated

1. Serological response by IPMA assay, at:

T_0 : bleeding and vaccination

T_{21} : bleeding and revaccination

T_{41} : bleeding

T_I : bleeding and infection

T_{I+7} : bleeding at 7 days post infection

2. Evaluation of rectal temperature, local reaction and

general reaction, during the 4 days posterior to vaccination and revaccination, or until the temperature or clinical signs, if any, disappear.

3. Evaluation of rectal temperature, feed intake and clinical signs during the 6 days following experimental infection.

4. Detection of antibodies in serum and isolation of virus in serum and in monocytes extracted from whole blood.

5. Reproduction parameters at the time of farrowing, such as the number of piglets born alive, number of stillborn or mummified piglets and number of piglets born alive but weak that died within the first week.

6. Determination of the amount of virus present in serum by means of titrations on macrophages based on CPE.

7. Determination of virus in pleural liquid and lungs of the piglets.

The reproductive parameter results are shown in Tables 11-12 (challenge with PRRS-CY-218-JPD-P5-6-91) and 13-14 (challenge with SDRP II 8B).

TABLE 11 (Vaccinated sows)

Challenge with PRRS-CY-218-JPD-P5-6-91

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
	37[a]	14					
	39	6	6	--	--	--	5
	68	12	8	2	1	1	7
	45	10	7	1	--	2	6
	19	10	9	1	--	--	8
	38	9	8	--	--	1	8
	41	7	5	--	--	2	5
	71	11	6	1	--	4	6
	36	10	6	1	1	2	6
	26[b]						
	TL	75	55	6	2	12	51

TL = Total

% of live piglets: 68% (75 born / 51 survive)

68% protection is observed when comparing the piglets born alive with those surviving 7 days of life. The fact that experimental infection is much more potent than natural infection in the field, added to the above results, make it possible to foresee that the prospects for protection are even better.

TABLE 12 (Unvaccinated sows)

Challenge with PRRS-CY-218-JPD-P5-6-91

(1)	(2)	(3)	(4)	(5)	(6)	(7)
63	9	--	--	1	8	--
88	6	--	3	--	3	--
79	8	--	3	--	5	--
22	12	2	--	--	10	1
28	7	3	--	--	4	3
TL	42	5	6	1	30	4

TL = Total

% of live piglets: 9.5% (42 born / 4 survive)

The Spanish strain used for infection has an extremely high pathogenic potency (90.5%) since only 4 piglets survived the first week out of the 42 piglets born.

TABLE 13 (Vaccinated sows)

Challenge with SDRP II 8B

(1)	(2)	(3)	(4)	(5)	(6)	(7)
51	13	10	2	--	1	10
27	14	12	1	--	1	11
16	9	8	--	--	1	7
17	15	13	--	--	2	12
1	7	--	--	--	--	7
8	15	13	--	--	2	13
57	10	9	--	--	1	8
61	12	8	1	--	3	8
78	10	10	--	--	--	10
52[c)]						
TL	105	83	4	--	11	86

TL = Total

% of live piglets: 82% (105 born / 86 survive)

82% approximate protection is observed

TABLE 14 (Unvaccinated sows)

Challenge with SDRP II 8B

(1)	(2)	(3)	(4)	(5)	(6)	(7)
62	12	7	1	4	--	9
81	15	13	--	--	2	13
4	12	12	--	--	--	11
94	14	9	1	--	4	9
1	13	8	1	4	--	8
TL	66	49	3	8	6	50

TL = Total

% of live piglets: 75.7% (66 born / 50 survive)

24.3% approximate mortality is observed. The pathogenicity of the French strain is very weak (24.3%) when comparing with the results obtained from the challenge with Spanish strain (mortality 90.5%). The results used for the evaluation of efficacy comparing vaccinated and unvaccinated sows are not very significant in the case of the French strain. However and in any case, pathogenicity in the vaccinated animals is reduced to 17.8%.

Key to Table 11 - 14

[a] : A different disease (not PRRS) (the piglets are not included)

[b] : Sick, died before infection

[c] : Sick, died by reason of another cause

(1) : Sow reference

(2) : Total number of piglets

(3) : Number of healthy piglets born

(4) : Number of weak piglets born

(5) : Number of live splay-legged piglets born

(6) : Number of stillborn piglets

(7) : Number of piglets alive after the 1st week

PATENT CLAIMS

1) A vaccine capable of preventing porcine reproductive and respiratory syndrome (PRRS), characterized on account of the fact that it comprises a suitable quantity of PRRS viral antigen or virus, Spanish strain, inactivated, as well as a suitable adjuvant, and optionally, a preserver.

2) Vaccine as per patent Claim 1, characterized on account of the fact that the said PRRS virus, Spanish strain, is the strain denominated PRRS-CY-218-JPD-P5-6-91, deposited at ECACC, with accession number V93070108.

3) Vaccine as per patent Claim 1, characterized on account of the fact that it contains a quantity of inactivated virus of, at least, $10^{5.5}$ $TCID_{50}$/dose.

4) Vaccine as per patent Claim 1, characterized on account of the fact that the said virus has been grown on a pig's lung alveolar macrophage culture.

5) Vaccine as per Claim 1, characterized on account of the fact that the said virus has been grown on a pig's lung alveolar macrophage and ST cell (ATCC CRL 1746 ST) co-culture.

6) Vaccine as per Claim 1, characterized on account of the fact that the said virus has been grown on ST cell (ATCC CRL 1746 ST) culture.

7) Vaccine as per patent Claim 1, characterized on account of the fact that the said virus has been grown on pig's lung alveolar macrophage hybrid cells fused with ST cells by means of hybridization.

8) Vaccine as per patent Claim 1, characterized on account of the fact that the said virus has been grown on pig's lung

alveolar macrophage hybrid cells fused with L-14 cell line (ECACC no. 91012317) or with cell line Jag-1.

9) Vaccine as per patent Claim 1, characterized on account of the fact that the said virus has been grown on ST cells or any other porcine cell line into which have been introduced the genes coding for pig's lung alveolar macrophage membrane receptors for the PRRS virus.

10) Vaccine as per patent Claim 1, characterized on account of the fact that the said adjuvant is an oily adjuvant.

11) Vaccine as per patent Claim 10, characterized on account of the fact that the said oil adjuvant is constituted by a mixture of Marcol (RTM) 52, Simulsol 5100 and Montanide (RTM) 888.

12) Vaccine as per patent Claim 1, characterized on account of the fact that it is an emulsion of (i) an aqueous antigenic phase containing the inactivated virus, and (ii) an oily phase containing the adjuvant.

13) Vaccine as per patent Claim 12, characterized on account of the fact that the said emulsion is composed of 53% by volume of an aqueous phase containing the inactivated virus and 47% by weight of an oily phase containing the adjuvant.

14) Vaccine as per any of the previous patent claims, characterized on account of the fact that it is capable of inducing cellular immunity in the vaccinated animal.

15) Vaccine as per patent Claim 14, characterized on account of the fact that it contains additionally cell response potentiation substances (CRP) that potentiate cell immune effect, such as IL-1, IL-2, IL-4, IL-5, IL-6, IL-12, g-IFN, cell necrosis factor and similar substances.

16) Vaccine as per patent Claim 1, characterized on account of

the fact that the adjuvant is an aqueous adjuvant.

17) Vaccine as per patent Claim 16, characterized on account of the fact that it contains additionally cell response potentiation substances (CRP) that induce cell immune effect, such as IL-1, IL-2, IL-4, IL-5, IL-6, IL-12, g-IFN, cell necrosis factor and similar substances.

18) Vaccine as per patent Claim 1, characterized on account of the fact that the adjuvant is an adjuvant that can modulate and immunostimulate cell response, such as MDP, ISCOM or liposomes.

19) Vaccine as per any of the previous patent claims, characterized on account of the fact that it is capable of avoiding return to service in the vaccinated animal.

20) A vaccine capable of preventing porcine reproductive and respiratory syndrome (PRRS) characterized on account of the fact that it is an emulsion comprising:

a) 53% of an aqueous phase containing PRRS viral antigen in DMEM culture medium, Spanish strain, inactivated, at minimum concentration of $10^{5.5}$ $TCID_{50}$/dose, and

b) 47% of an oily phase containing a mixture of Marcol(RTM) 52, Simulsol 5100 and Montanide(RTM) 888.

21) Vaccine as per patent Claim 20, characterized on account of the fact that the said viral antigen is the PRRS virus, Spanish strain, denominated PRRS-CY-218-JPD-P5-6-91, deposited at ECACC, with accession number V93070108.

22) Vaccine as per any of patent Claims 20 or 21, characterized on account of the fact that it is capable of inducing cell immunity in the vaccinated animal.

23) Vaccine as per patent Claim 22, characterized on account of the fact that it contains additionally cell response potentiation substances (CRP) that potentiate cell immune effect, such as IL-

1, IL-2, IL-4, IL-5, IL-6, IL-12, g-IFN, cell necrosis factor and similar substances.

24) Vaccine as per any of patent Claims 20 to 23, characterized on account of the fact that it is capable of avoiding return to service in the vaccinated animal.

25) A bi-or multivalent vaccine capable of preventing porcine reproductive and respiratory syndrome and another or other porcine infections, characterized on account of the fact that it contains a suitable quantity of PRRS viral antigen or virus, Spanish strain, inactivated, plus one or more porcine pathogens.

26) Vaccine as per patent Claim 25, characterized on account of the fact that the said viral antigen is the PRRS virus, Spanish strain, denominated PRRS-CY-218-JPD-P5-6-91, deposited at ECACC, with accession number V93070108.

27) Vaccine as per patent Claim 25, characterized on account of the fact that it includes, at least, one porcine pathogen selected from the group made up of Actinobacillus pleuropneumoniae, Haemophilus parasuis, Porcine parvovirus, Leptospira, Escherichia coli, Erysipelothrix rhusiopathiae, Pasteurella multocida, Bordetella bronchiseptica, Porcine respiratory coronavirus, Rotavirus, or against the pathogens causative of Aujeszky's disease, swine influenza or transmissible gastroenteritis.

28) A procedure for the preparation of a vaccine capable of preventing porcine reproductive and respiratory syndrome (PRRS), containing a suitable quantity of PRRS virus, Spanish strain, inactivated, plus a suitable adjuvant and, optionally, a preservative, characterized on account of the fact that it comprises:

1) the growing of the causative virus of PRRS, Spanish strain, in a suitable cell system,

2) the collection of the virus from the said cell system when minimum titre of $10^{5.5}$ $TCID_{50}$/ml has been attained,

3) the inactivation of virus by means of physical or chemical methods, and

4) the mixing of the inactivated virus with the adjuvant and the preservative.

29) Procedure as per patent Claim 28, characterized on account of the fact that the said PRRS virus, Spanish strain, is the virus denominated PRRS-CY-218-JPD-P5-6-91, deposited at ECACC, with accession number V93070108.

30) Procedure as per patent Claim 28, characterized on account of the fact that the said virus has been grown on:

i) pig's lung alveolar macrophage culture, or on

ii) a co-culture of pig's lung alveolar macrophages and ST cells (ATCC CRL 1746 ST), or on

iii) ST cell culture (ATCC CRL 1746 ST), or on

iv) pig's lung alveolar macrophage hybrid cells fused with ST cells, or on

v) pig's lung alveolar macrophage hybrid cells fused with L-14 cell line (ECACC no. 91012317) or with cell line Jag-1, or on

vi) ST cells or on any other porcine cell line into which have been introduced genes coding for pig's lung alveolar macrophage membrane receptors for the PRRS virus.

31) DNA sequence of the virus causative of PRRS, Spanish strain, comprising essentially the DNA sequences shown in Figures 1 to 5.

32) Virus causative of PRRS, Spanish strain, whose characteristics essentially correspond to those of the virus denominated PRRS-CY-218-JPD-P5-6-91, deposited at ECACC, with accession no. V93070108.

33) Virus as per patent Claim 32, inactivated, capable of being put to use in the formulation of vaccines capable of preventing porcine reproductive and respiratory syndrome.

34) Virus as per patent Claim 32, inactivated, capable of being put to use in the formulation of bi-or multivalent vaccines capable of preventing porcine reproductive and respiratory syndrome and other porcine infections.

Patents Act 1977 **Examiner's report to the Comptroller under Section 17** (The Search report) 56	Application number GB 9418775.4
Relevant Technical Fields (i) UK Cl (Ed.M) C3H (HB4B); C6F (FJ); A5B (BAA) (ii) Int Cl (Ed.5) A61K 39/12; C07K 15/04, 15/12; C12N 7/02, 15/40	Search Examiner MR C SHERRINGTON Date of completion of Search 9 JANUARY 1995
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